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On the Protein Metabolism of Tissue Cells *in Vitro*[1,2]

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Knowledge of the protein metabolism of the organism was, until a few years ago, rather limited; nutritional proteins were known to be broken down to amino acids in the alimentary canal, where they were taken up by the blood. Certain blood proteins were known to be built up in the liver. But the further fate in the organism of the proteins was obscure.

The fact that nutritional proteins were broken down in the alimentary canal was recognized at an early period, but not until much later did the first experimental evidence appear. Loewi (*1*), Henriques and Hansen (*2*) and Abderhalden, Baumann, and London (*3*) found that the administration of completely hydrolyzed proteins kept animals in nitrogen equilibrium. Investigations by Henriques and Andersen (*4*) proved beyond doubt that it was possible to maintain nitrogen equilibrium by intravenous administration of the protein constituents. Proteins were presumed to be rebuilt in the intestinal wall, since it had been impossible to find the split products in the blood.

The amino acids in the blood were first discovered by Van Slyke and Meyer (*5-7*) in 1912, and by Abel *et al.* (*8*) in 1913.

These findings represent the second milestone in the studies on protein metabolism. Amino acids are always present in blood, even in the fasting periods, and are always deposited in the organism as body proteins, not as amino acids.

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[2] This material was presented as a speech delivered by invitation at the National Cancer Institute in August 1952, reviewing the work on nutrition of tissue cells *in vitro* done in the institute at Copenhagen during the past ten years.

The findings of Rose (*9*) represent the third milestone in the studies on protein metabolism in the organism. In 1938 this author found as few as ten different amino acids to be sufficient to maintain life in higher animal organisms, and even sufficient to produce growth. Hence, an organism must have a capacity to produce all other amino acids from the ten contained in Rose's mixture, which consists of valine, leucine, isoleucine, threonine, lysine, tryptophane, histidine, phenylalanine, methionine, and arginine.

In 1937 Braunstein and Kritzmann (*10*) observed a process by which the organism might develop certain new amino acids. By the interaction of an enzyme (a transaminase), glutamic acid, among others, was capable of transferring an amino group to a ketonic acid. In this way a new amino acid was produced.

Finally, the excellent works of Madden and Whipple (*11*) and Schoenheimer (*12*) should be mentioned. By using amino acids labeled with isotopes, they showed proteins to be far more reactive than hitherto presumed. They showed that the nitrogen groups in the tissue proteins constantly underwent chemical changes; peptides underwent cleavage; and amino acids were liberated.

Whereas carbohydrate metabolism in the tissue cells is well understood, this does not apply to the protein metabolism. Knowledge of the protein structure is still inadequate, since the proteins are much more complicated in structure than the carbohydrates. As opposed to carbohydrates, which mainly serve as sources of energy, the main function of the proteins is to contribute building material for new protoplasm and to enable the synthesis of the highly different proteins essential to the many different cell types and their specific metabolites.

As mentioned above, it is a fact that proteins are hydrolyzed in the alimentary canal and ultimately appear in the blood as amino acids and polypeptides; it is also a fact that the nitrogen equilibrium in animals is maintained when amino acids are supplied intravenously. The tissue cells must thus have the capacity of utilizing nitrogen in this form.

Certainly, tissue cells were presumed to build up their protoplasm from amino acids in the blood, but any further knowledge of this process was lacking because, until quite recently, it had not been possible to prove it by means of tissue cultures—the only adequate experimental approach. So far, the results of all experiments in that direction have been the same in that the findings were negative. Amino acids were found to be either toxic or, at least, nonessential to the maintenance of cellular life. In his recent report on protein synthesis the American protein-chemist Alcock (*13*) said, "The tissue culture, the only case of protein synthesis under any measure of control, carries out its work as secretly as the whole animal, and has so far yielded no information."

It was a matter of great moment to obtain the unequivocal proof of the cellular capacity for growth and proliferation in a medium in which amino acids represented the source of nitrogen. Experiments to this effect were instituted by Burrows and Neymann (*14*) in 1917. They culti-

vated tissue cells in mixtures, the constituents of which were either pure amino acids or hydrolyzed proteins, but they found nothing except that the amino acids were toxic. The question was later reconsidered by Carrel and Baker (*15*) who examined the effects of glycine, alanine, leucine, phenylalanine, cystine, asparagine, aspartic acid, arginine, lysine histidine, proline, oxyproline, prolylglycine, and tryptophane, in mixtures containing nucleic acids, iodine, and various metal salts. On the basis of their experiments the conclusion was drawn that amino acids were equally incapable of prolonging the life and accelerating the growth of the cells. It was evidently the authors' concept that acceleration of cellular growth would result from the supplementation by amino acids. When the acceleration failed to occur it was concluded that the amino acids were ineffective. That the acceleration failed to occur is not surprising since a sufficient quantity of amino acids was already present in the blood plasma and serum used as culture medium. It is, indeed, not justifiable to expect an accelerated rate of growth as a consequence of an increase in the already physiologically adequate amino-acid concentration in the blood.

Such were conditions when our experiments were instituted about ten years ago.

If amino acids were essential to the metabolism of the tissue cells, their removal from the culture medium must imply an inadequacy; this presumption, which proved to be correct, was the basic idea in our experiments. When all components in the culture media composed of blood plasma and serum, as well as the embryonic tissue extracts, were dialyzed, the medium was found to be inadequate to maintain cellular life. Within 24 hours all cells in such a culture medium died.

The basic experiment was as follows: If each of the two halves of a tissue fragment was placed in a separate flask, one containing normal culture medium and the other containing the medium that had been dialyzed, a complete autolysis of the cells in the dialyzed medium occurred, whereas cells in the normal medium continued their normal growth. The condition of inadequacy of the dialyzed medium was sufficient to prevent any maintenance of life. Hence, a deficiency in the basic building materials made the tissue cells unable to attack and utilize the higher blood proteins. The same applied to such tissue cells as intestinal epithelial cells, chorionic cells, and highly proteolytic tumor cells, whose normal function is to hydrolyze large quantities of proteins.

Preliminary experiments disclosed beyond doubt that the serum dialysate and ultrafiltrate contained all the components necessary for complementing the inadequate, dialyzed plasma medium. Thus, the path was paved for the performance of an exact analysis on such substances as may convert an inadequate medium into an adequate one.

The importance of amino acids was subjected to further examination in extensive experiments where mixtures of different amino acids were tested. Mixtures with from ten to fifteen different acids proved, however, hardly more efficient than mixtures with only a few acids, provided that

cystine was always included. The effect of cystine alone proved to be brief and not very pronounced, but definitely supplementary. In the course of numerous experiments on various combinations a satisfactory effect was achieved with a choice of nine amino acids, particularly if mixed in the proportions in which they are found in the quantitative analysis of fibrin by Bergmann and Niemann (*16*). The components of the mixture were: lysine, arginine, tryptophane, methionine, histidine, glutamic acid, aspartic acid, proline and cystine. The mixture was able to prolong cellular life considerably in the dialyzed plasma medium. Considerable proliferation occurred on the further addition of high-molecular, embryonic growth substances, and a culture of considerable size actually developed.

The above findings represent the first proof of the capacity of tissue cells to grow and to synthesize new protoplasm from the basic protein components. But the experiments revealed other aspects worthy of notice. The relative concentration of the individual amino acids was evidently highly important. A mixture containing equal proportions by weight of the acids proved almost ineffective. As, for example, there is an optimum level for sugar in the blood, it would not be unreasonable to suggest that there may be a certain optimal level in the blood for each individual amino acid. There may be some relationship between the contents of amino acids in the fibrin and in the protoplasm as an indication of a parenteral digestion of fibrin. It is hardly probable that the amino acids in the blood should be exclusively and directly derived from intestinal absorption, particularly not during starvation periods when great amounts of proteins from the blood and cells presumably are hydrolyzed.

All the amino acids in Rose's mixture (*9*) are claimed to be vital to the organism. The mixture deviates from the Bergmann mixture by containing methionine but no cystine. Rose's mixture proved, however, entirely ineffective on tissue cells in a dialyzed culture medium [Fischer (*17*)].

One of the most important discoveries made from these experiments was the vital position of cystine, and the inability of the tissue cells, as opposed to the organism, to demethylate methionine. If, for example, cystine were removed from the Bergmann mixture, the remaining eight acids proved unable to complement the inadequate dialyzed medium, and the cells autolyzed as rapidly as in the pure dialyzed plasma medium. Cystine is actually the only acid capable of compensating for a while for some of the inadequacy of the medium, but the effect was more pronounced with the application of the entire Bergmann mixture. If the natural cystine was replaced with *d*(−)-cystine, the mixture proved entirely ineffective.

The vital position of cystine is exclusively ascribed to the sulfur groups. But other sulfur-containing components were able to replace cystine. These included ovalbumin and serum albumin after denaturation, denatured insulin, and even Na_2S in very low concentrations. The effect of all the various substances was due to the fact that they contained disulfide and thiol groups, respectively.

Although life can be maintained and even growth induced, as well as

new protoplasm synthesized, by the use of Bergmann's mixture in tissue cultures, the size and appearance of the cells are far from being identical with those in a nondialyzed medium. Important components were still lacking.

Not only cystine but also lysine and glutamic acids proved to be indispensable to the tissue cells. The specific position of glutamic acid in the process of amination and deamination might indicate that its main purpose is as a carrier of NH_2 groups for the production of other amino acids.

Also, dialyzed plasma proteins digested by pepsin and erepsin had a considerable supplementary effect on the culture medium without, however, creating normal growth conditions [Fischer (*18*)]. A pronounced difference was found in the effects of digested homologous and heterologous plasma proteins. This was most evident in the effect of the polypeptides, probably because the structure of homologous polypeptides for all practical purposes corresponds to the structure of the cellular protein. But polypeptides alone were not capable of exciting normal growth conditions in the tissue cells. Some important constituents were still lacking. These are probably cofactors to energy-yielding systems and activating substances in enzyme processes in the cells. Attempts at supplementing the amino acids with all known vitamins and glutathione were equally unsatisfactory. The inadequate medium was, however, supplemented almost completely if dialyzable substances from extracts of yeast or from green malt were administered.

Low-molecular substances derived from such different substances as yeast, barley malt, and fish sperm were thus found to be closely related to those found in the intercellular spaces in higher animal organisms (*19–23*).

Large quantities of dialysates from calf embryo were collected, vacuum-dried at 40 to 50° C., and subsequently treated with a mixture of glacial acetic acid and methanol. By this process the majority of the inorganic salts sedimented in the form of chlorides, whereas the active substances remained in the solution. After centrifuging, the substances were precipitated by adding three volumes of absolute alcohol; the sediment was isolated by centrifuging, and treated with absolute alcohol and ether; 0.2 ml. of the dialysate with a concentration per ml. of 10 to 33 mg. when added to a Carrel flask was sufficient to produce normal growth (*24–26*).

Barium acetate precipitated all active components together with the phosphate. On dissolution of all sediments and on removal of the barium, all inorganic phosphate could be precipitated with calcium without any decline in activity. By various methods very active fractions were obtained, but the active components were not isolated or identified.

With a view to studying the carbohydrate metabolism in the cells, dialyzed culture media, supplemented with the necessary accessory growth factors except sugar, were examined. *Tissue cells in the sugar-free medium died as rapidly as in the nonsupplemented, dialyzed plasma medium*, and such kinds of sugar as might be utilized by the tissue cells were readily determined. The following varieties of sugar were examined: saccharose,

lactose, glucose, mannose, galactose, arabinose, xylose, amylum, glycogen, *d*-lactic acid, and pyruvic acid; but only glucose, fructose and mannose could be utilized by the cells (*27*).

Low-molecular growth factors were designated as accessory factors. This was done so as not to confuse them with the high-molecular growth factors, or the nucleoproteids in extracts of embryonic tissues, which accelerate cell division. Such nucleoproteids are without any effect on tissue cells when no accessory factors are present.

The increase of the surface area of the culture as well as the morphology of the cells served as indication of the effect, on the tissue cells, of the various diets containing low-molecular substances.

The more or less pronounced changes in the cells, characteristic of undernourished cells, were porportionate to the inadequacy of the culture media. The cells decrease in size and get thinner. Simultaneously changes occur in their various organs (*28*). The occurrence of one or two juxtanuclear fatty vacuoles was typical. The change occurred when the inadequacy was due to a shortage in one or more of the vital substances, or when the concentration of the normally present low-molecular-substance groups was reduced to 25 percent or less of its normal concentration. Figures 1 to 3 illustrate the action of the various media on the cells.

It has already been observed that cells in an inadequately supplemented dialyzed medium were much smaller than those in a normal medium. The mitochondria reacted promptly when external conditions deviated from the normal. They disintegrated into small grains much resembling a string of pearls or a row of beads. Natural red granules under normal conditions resemble small grains, but develop into large, coarse vacuoles under abnormal conditions.

All morphologic changes in the cells could be ascribed to the inadequacy of the medium, either because one or more of the vital substances were lacking, or because of an insufficient concentration of dialyzable substances.

A basal diet was subsequently composed containing various groups of such substances as are known to be important to the organism (*25*, *26*), and we were successful in producing a complex neutral solution as recorded by G. Ehrensvärd (see table 1). One ml. of this medium when added to the dialyzed plasma medium in Carrel flasks caused the cells to grow readily in this medium, but they were rather atrophic and resembled tiny glass splinters (fig. 4).

On addition of glutamine the cells showed a normal appearance. When the organic phosphate was removed from the diet mixture a pronounced inadequacy set in, an occurrence which was also manifest when all amino acids were removed from the diet.

Certain metallic salts known for their catalytic effect in enzymatic processes, as well as adenosine triphosphate, cozymase, the various vitamins, choline, creatine, and C_4-acids active in the Krebs cycle, were among the substances which were nonessential to cellular life and growth in *short-term experiments*.

The normal growth of tissue cells, even in the absence of the above

substances, was a surprise to us. The explanation may be that some of the low-molecular substances were retained by the dialyzed plasma bound to the proteins. It is generally admitted that heavy metal ions and certain organic substances such as vitamin B_6 are not readily dissociated from protein.

TABLE 1.—*Mixture V-605 (mg. of substances contained in 1 liter of solution)*

$NaCl$	7,500	*l*(+)-Lysine, 2 HCl	15
KCl	200	*l*(−)-Histidine, HCl	5
$CaCl_2$	200	*l*(+)-Arginine	2
$MgCl_2$	100	*d,l*-Valine	14
Na_2HPO_4	50	*l*(−)-Leucine	9
$NaHCO_3$	1,000	*d,l*-Isoleucine	10
		d,l-Threonine	12
$FeCl_2$	0. 6	*d,l*-Phenylalanine	7
$CuCl_2$	0. 2	*l*(−)-Tryptophane	2
$MnCl_2$	0. 3		
$ZnCl_2$	1. 0	*d,l*-Methionine	6
$CoCl_2$	0. 01	Choline (as Hydrochloride)	10
		Creatine	10
Glucose	800	Nicotinic acid	0. 3
Mannose	100		
Galactose	100	Cystine	5
Inositol	20	Glutathione	5
Adenosine triphosphate	200	Pantothenic acid	0. 07
Fructose diphosphate	100	Biotin	0. 007
β-Glycerophosphate	100		
Inosinic acid	30	*p*-Aminobenzoic acid	1
		Hypoxanthine	100
Cozymase	5		
Thiamine	3	Sodium succinate	10
Riboflavin	0. 2	" fumarate	10
Pyridoxine	0. 3	" malate	10
		" oxaloacetate	10
Glutamine	250		
		Ascorbic acid	2
		Methylnaphthohydroquinone-sulfate	0. 005

The significance of glutamine was anticipated because of its function as a growth factor for certain streptococci and because of its position in the glycolysis of certain streptococci as recorded by McIlwain. The significance of fructose diphosphate is obvious, analogous as it is to its effect on the process of fermentation; its purpose is to supply the glycolytic system with sufficient quantities of hexose diphosphate in the period following the explantation of the tissue before the cells regain their normal enzymatic functions. The possibility of removing from the embryonic tissue extract all organic phosphate from the accessory growth factors in the dialysate without any interference in the biologic activity should also be emphasized in this connection. *No* glutamine is contained in the above products and their effect may be different from that of the synthetic diet. The complicated mixture of table 1 could be reduced to the simpler mixture of table 2. The least complicated diet on which cellular life in the dialyzed plasma medium may be maintained includes fructose diphosphate, glutamine, and glutathione.

TABLE 2.—*Mixture V-614*

	(*Mg.*)		(*Mg.*)
NaCl	7, 500	*l*(+)-Arginine	4
KCl	200	*d,l*-Valine	28
$CaCl_2$	200	*l*(−)-Leucine	18
$MgCl_2$	100	*d,l*-Isoleucine	20
Na_2HPO_4	50	*d,l*-Threonine	24
$NaHCO_3$	1, 000	*d,l*-Phenylalanine	14
		l(−)-Tryptophane	4
Glucose	2, 000		
Fructose diphosphate	200	Cystine	10
		Glutathione	10
l(+)-Lysine, 2HCl	30		
l(−)-Histidine, HCl	10	Glutamine	250

Based on long experience, we have now composed a synthetic diet on which heart fibroblasts are cultivated in coverslip cultures throughout ten passages or more. In this medium cells display completely normal conditions, even as to structure.

The constituents of the synthetic diet were as follows:

10 ml. dialyzed blood plasma
1 ml. of a mixture including glutamine, fructose diphosphate, and glutathione
1 ml. 2 percent glucose
1 ml. trypsin-digested, dialyzed blood plasma. or a synthetic mixture of amino acids
0.5 ml. glycine.

In this connection it should be observed that glycine is among the amino acids essential for the fowl.

The above diet does not merely maintain cellular life, but also enables the cells to synthesize new protoplasm. This is demonstrated in figures 5 and 6. During these studies a convenient technique was developed for obtaining a single layer of cells suitable for microscopy (*29*).

The experiments here dealt with represent, of course, only a fraction of the work carried out during the last ten years, during which time about 20,000 cultures were used. Experiments in this field are certain to be gratifying and may even result in the discovery of other active substances, such as vitamins, hormones, and cofactors of various kinds in the intermediary cellular metabolism. They may bring forward new information on the biologic processes occurring in the cells during their differentiation and organization, and may even, at some future date, make it possible to register the specific diet characteristic of the various types of tissue cells. One thing is already clear, namely that one diet, which may be satisfactory for one type of cell, may be inadequate for another. Cardiac myoblasts are, for example, unable to survive on a diet that is ideal for liver fibroblasts. Also, a certain diet has a selective effect on the large wandering cells in the tissue, the so-called macrophages. In a mixed culture of certain connective tissue cells and a few macrophages, the connective tissue cells were gradually destroyed, leaving a pure culture of macrophages. This is demonstrated in figure 7.

More than once it has occurred to us that amoeboid macrophages growing side by side with fibroblasts in a medium inadequate for cardiac fibroblasts may even secrete materials by which the medium regains its adequacy. This observation supports the theory that macrophages are trephocytes, that is, cells supporting other cells; this discovery was made by Carrel many years ago. This quality in the macrophages is an indication of such cells being less specialized than the fixed cells. Autonomically, macrophages may be in closer relation to an animal organism than any other integrating cell types in the intact organism. The demonstration of the trephocytic activity makes it possible to learn more about the chemical character of the trephones.

By now it is generally admitted that the concentration of all amino acids in the blood, as well as the concentration of the individual acids in the mixture, are stable throughout periods of fasting and starvation. If tissue cells are supposed to build up their protoplasm from the said acids, a very intricate control of every individual vital amino acid in the blood is thereby anticipated. The organism might solve the problem by the application of but one single mechanism, provided that all tissue cells are capable of digesting one or more of the proteins in the blood. Fibrinogen, among others, might be the protein supplying the majority of the amino acids, which are consequently present in the blood in a quantity proportionate to the requirements of the cells. A control of one substance such as fibrinogen requires a much less complicated mechanism than a control of every single amino acid. However, the maintenance of a stable concentration of the amino acids in the blood might also be due to a dynamic balance or equilibrium between breaking down and synthesis.

The proteolytic enzymes involved in the process of digestion may be either desmo-enzymes on the cellular surface, or proteolytic enzymes in the blood plasma, or both. The capacity of somatic tissue cells to break down proteins is incontestable and is clearly demonstrated in experiments by Santesson (*30*), who found slices of coagulated chicken protein to be digested directly below the fragment of tissue in the cultures. However, the experiments rather indicate a contact digestion, brought about by enzymes on the cellular surface.

A series of inactive enzymes, which may be activated in different ways, are present in the blood plasma. One is the plasmatrypsin complex, the designation of which is now plasmin. According to Schmitz (*31*, *32*) this particular enzyme is bound to low-molecular inhibiting substances. This result is similar to the findings of Northrop in the case of trypsinogen and chymotrypsinogen. In the blood, a kinase is also present which, by blocking the inhibitors, exerts an activating effect on the enzymes. By activation of the enzymes and absorption to purified fibrin, Schmitz proved among other things a breaking down into low-molecular products.

The capacity of tissue cells to break down proteins in the culture medium was further established in other ways. Cardiac myoblasts, cultured in a medium containing mainly fibrin, will in the course of 24 hours lyse the said medium.

Other experiments revealed the digestion to be a result of activated proenzymes in the blood plasma (*33*). No visible digestion is produced by chicken sarcoma cells in heterologous rabbit plasma, not even when rabbit serum is added. But when rabbit serum shaken vigorously with sand is added to rabbit plasma, the sarcoma cells display a digestive activity similar to the one occurring in homologous chicken plasma. The tryptic enzyme complex in the serum was activated by shaking. This is a process similar to the spontaneous, cellular activation process in a homologous plasma medium. As a further proof of the correctness of the theory, the following experiment is recorded: The addition to rabbit plasma of homologous chicken serum induced a digestion of rabbit plasma by the chicken tumor cells. But when homologous chicken serum was added after incubation at 56° C. for 1 to 4 hours, the enzymes in the serum were destroyed, and no digestion occurred.

The findings suggest that the proteolytic activity in the heterologous medium is a consequence of a cellular activating effect on the tryptic enzyme complex in the blood plasma. Thus, there is hardly any basic difference in the extensive digestive activity of tumor cells in the homologous blood plasma, and the almost invisible, and much more moderate activity of the normal cells. The proteolytic activity could be depressed by addition of the soybean inhibitor (*34*).

Although our understanding of the protein metabolism in the organism is still very defective, many new and important experimental facts are now in hand which open possibilities of postulating the mechanism of protein metabolism.

The main factors may be summarized as follows: Besides the external mechanism of digestion in stomach and intestines, where nutritional proteins are broken down into amino acids by the organism and converted into homologous protein, the organism contains a different system which might be designated "the internal alimentary canal," in which homologous protein is metabolized by the tissue cells in order to cover their requirements for the synthesis of new protoplasm.

In continuation of these investigations on the nutrition of tissue cells *in vitro* we have started using radioactive carbon C^{14} (*35*).

In the first experiments we have used glucose labeled with C^{14} in the cultivation of cells in normal media in order to find the amino acids in which carbon atoms from the glucose could be incorporated.

Radioactivity was found in appreciable amounts in the following acids: aspartic acid, glutamic acid, alanine, serine, glycine, and proline. The highest values were found in *aspartic acid* and *alanine*. These labeled amino acids all belong to the acids designated as nonessential by Rose. Our experiments show that some of these nonessential amino acids also in the tissue culture can be produced by the cells partly from other sources than preformed amino acids, though the amino group must by transamination be supplied by another amino acid, such as aspartic acid or glutamic acid.

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PLATE 140

FIGURE 1.—Heart fibroblasts (chicken) in a normal plasma medium. Stained with Hansen's hematoxylin.

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PLATE 141

FIGURE 2.—Heart fibroblasts in a dialyzed medium. The cells contain numerous large vacuoles and are beginning to disintegrate.

2

PLATE 142

FIGURE 3.—Heart fibroblasts in a dialyzed plasma medium containing 25 percent of a dialysate from blood serum.

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PLATE 143

FIGURE 4.—Heart fibroblasts in dialyzed medium showing insufficient compensation resulting in cells of the "glass-splinter type."

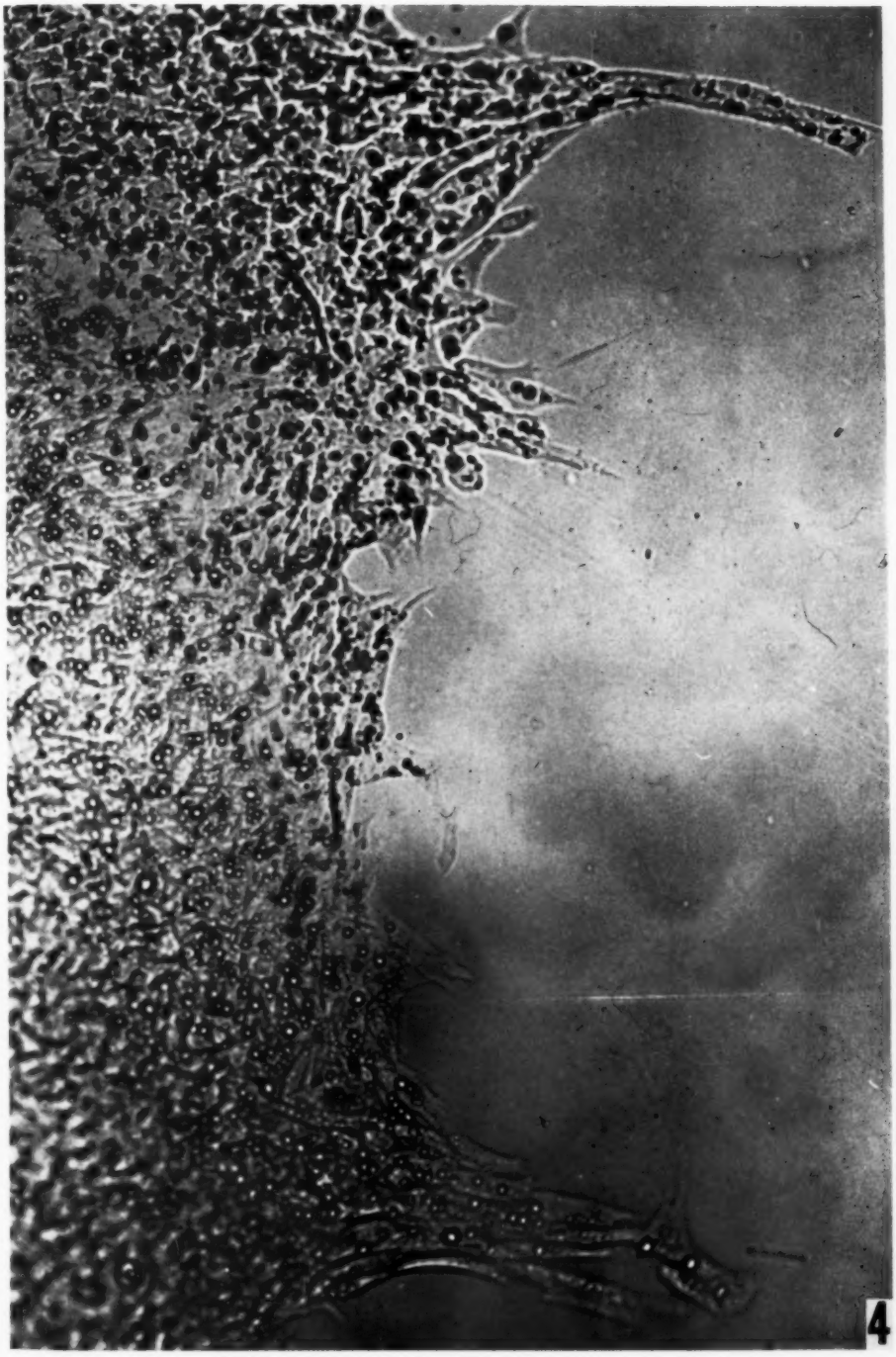
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PLATE 144

FIGURE 5.—Heart fibroblasts in dialyzed medium supplemented with glutamine, fructose diphosphate, glutathione, glycine, and a tryptic digest of ox fibrin.

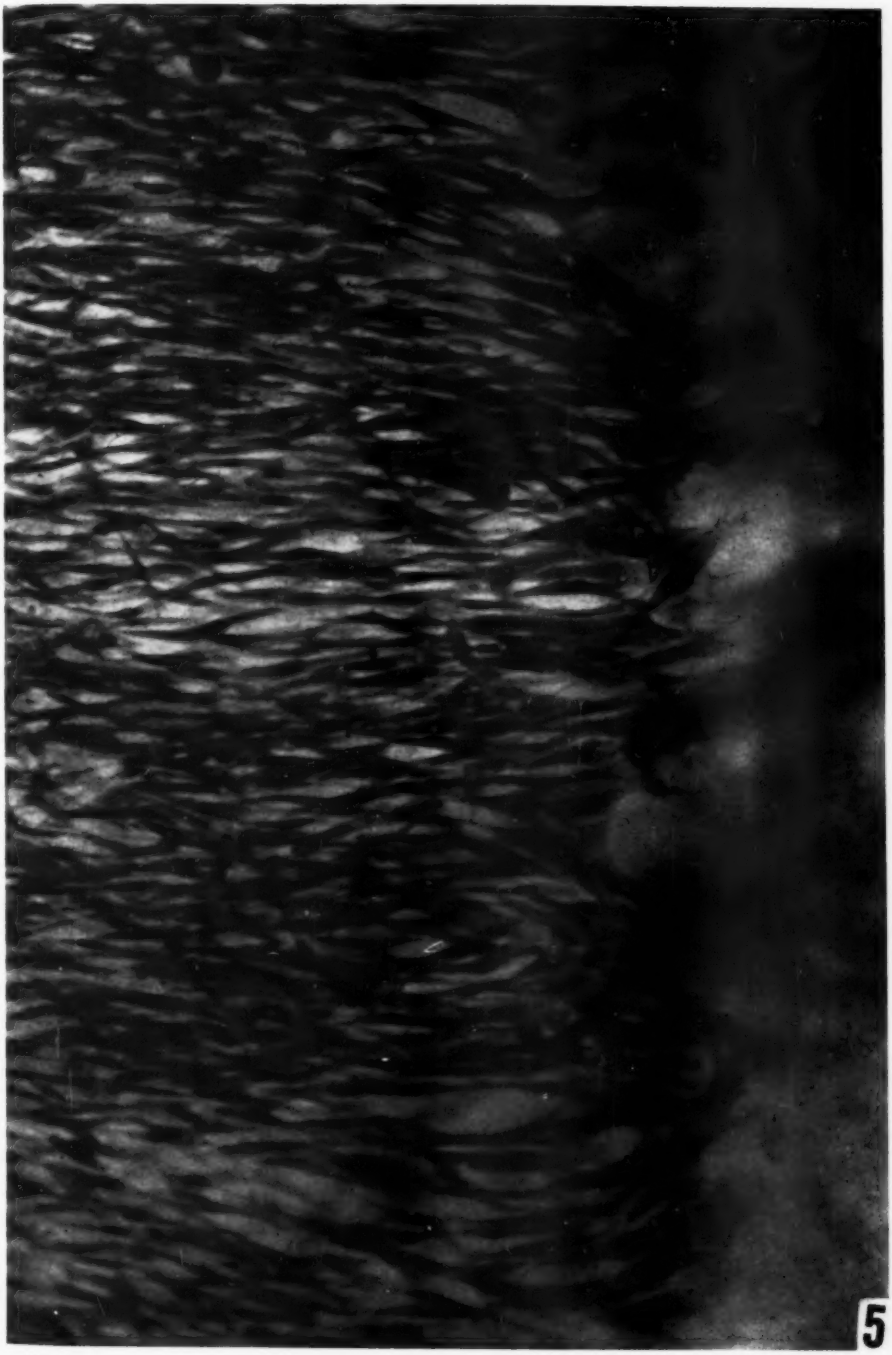
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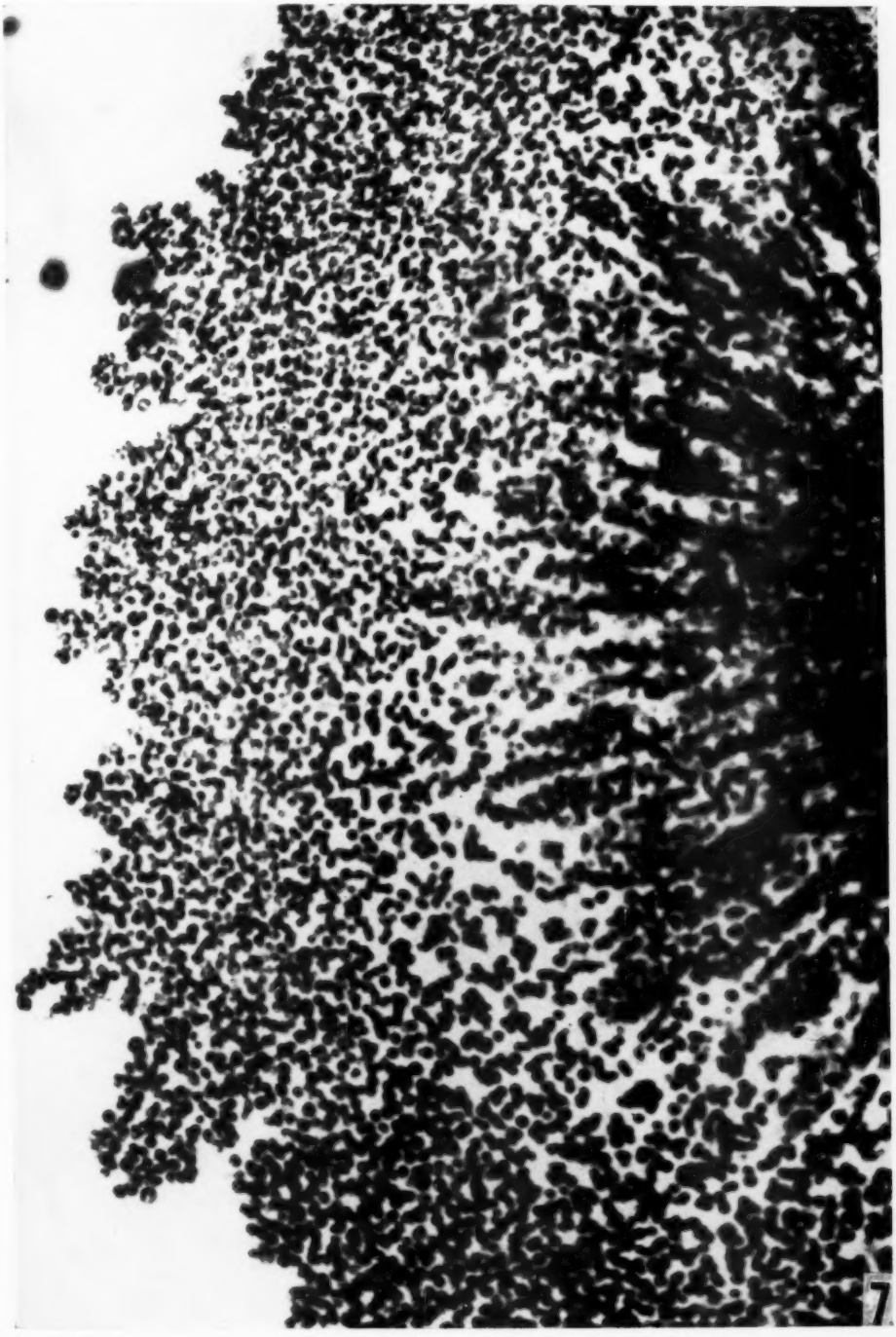
PLATE 145

FIGURE 6.—Heart fibroblasts in dialyzed medium supplemented with glutamine, fructose diphosphate, glutathione, glycine, and a tryptic digest of ox fibrin.

6

PLATE 146

FIGURE 7.—A culture of macrophages developed during activation.



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247481—53——6

Desoxypentose Nucleic Acids and Nucleoproteins of Malignant Tissues. I. The Nucleohistone of a Transplantable Mouse Lymphoma[1]

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Recent developments in knowledge of the chemistry of desoxypentose nucleic acids emphasize the complexity and multiplicity of this class of substance. Although numerous determinations have been made of the amount of DNA and PNA[3] in many normal and malignant tissues (*1*, *2*) relatively little is known of the physical and chemical character of the nucleic acids of different tissues. The investigations of Chargaff's group [reviewed in (*3*)] provide evidence that the composition of DNA is characteristic of the species from which it is derived, but that within the limits of present analytical methods the DNA of different tissues of the same species have the same over-all composition. As they point out, however, this result does not rule out the possibility that there may be differences in the sequence of arrangement of the nucleotides among nucleates of the same over-all composition. In addition, the possibility of highly unique configurations by virtue of branching or cross linking of the polynucleotide chains must be considered. It is to be expected that any such differences in sequence or configuration would be reflected in the physico-chemical behavior of the nucleates and of the nucleoproteins from which they are derived. However, much more information regarding the physico-chemical properties of nucleates derived from different sources, including growing, nongrowing, normal, and malignant tissues, is needed before these questions can be answered.

We are carrying out in this laboratory an investigation of the desoxypentose nucleic acid and the nucleoproteins of the transplantable lymphoma L1 (*4*) of the strain A mouse. This tumor was considered suitable for such studies because of its high content of DNA, rapid growth, relative freedom from necrosis, and uniformity of cell type. It is intended that these studies serve as a first step in an eventual comparison of the properties of these components with those of normal tissues of the same

[1] Received for publication November 19, 1952.
[2] National Institutes of Health, Public Health Service.
[3] DNA is used here as an abbreviation for desoxypentose nucleic acid, and PNA for pentose nucleic acid.

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species, and that they provide a baseline for investigation of the action on these components of such agents as radiation, mutagens, and chemotherapeutic compounds. They also contribute to the general problem of the extent to which the desoxypentose nucleic acids of different sources resemble or differ from each other.

The present paper deals with the isolation and partial physico-chemical characterization of the nucleohistone of the lymphoma; other papers in this series will be concerned with studies of the highly polymerized DNA that was prepared from this nucleohistone.

In defining the properties of the DNA or nucleohistone of a given tissue it is important that the isolation be as quantitative as possible and that degradation be reduced to a minimum. Losses in preparation might yield a product whose properties were not representative of the whole, while degradation by such agents as acid, alkali, enzymes, or heat might destroy some of the unique properties that would distinguish the particular substances. There will be presented below, *a*) a determination of the content of DNA and PNA of several generations of the tumor, *b*) a description of the method by which essentially all of the DNA can be isolated as the nucleohistone, and *c*) studies of the solubility, viscosity, and anomalous titration properties of the nucleohistone which indicate that its nucleic acid component is in an undenatured and highly polymerized state.

Experimental Results

Materials.—Strain A mice were used; the tumor was the transplantable lymphoma L1 whose history has been described elsewhere (*4*). The animals were killed and the tumors harvested about 20 days after transplantation.

Content of nucleic acids.—The determination of DNA and PNA was carried out by the method of Schneider (*5*), using the diphenylamine reaction for DNA and the orcinol reaction for PNA. The data for seven different generations of the tumor are given in table 1.

TABLE 1.—*The nucleic acid content of transplanted mouse lymphoma*

Preparation number	Nitrogen	Phosphorus	DNA Phosphorus*		PNA Phosphorus*		Recovery of DNA P as nucleohistone	N/P ratio
	mg./gm. fresh tissue	*mg./gm. fresh tissue*	*μg./mg. N*	*mg./gm. fresh tissue*	*μg./mg. N*	*mg./gm. fresh tissue*	*mg./gm. fresh tissue*	
1	22. 7	.....	66. 3	1. 50	12. 2	0. 275	.....	.....
2	24. 1	3. 68	58. 0	1. 38	17. 0	0. 415	1. 33	4. 1
3	24. 1	3. 85	53. 5	1. 27	21. 6	0. 529	1. 16	4. 4
4	29. 7	4. 63	47. 2	1. 40	14. 8	0. 438	.....	.....
5	28. 5	4. 15	49. 5	1. 41	13. 3	0. 379	.....	.....
6	24. 7	3. 97	47. 0	1. 16	16. 1	0. 397	0. 96	4. 3
7	24. 0	4. 08	58. 0	1. 39	18. 8	0. 453	1. 29	3. 8
Mean	25. 4	4. 06	54. 2	1. 36	16. 2	0. 412		
S. D.†	±2. 6	±0. 33	±6. 8	±0. 11	±3. 2	±. 078		

* The phosphorus was calculated from pentose measurements.

† S .D. = $\sqrt{\Sigma(d^2)/n-1}$

Isolation of Nucleohistone

Mirsky and Pollister (*6*) have described a method for the isolation of nucleohistone from animal tissues which depends on the remarkable differences in the solubility of nucleohistone in salt solutions of different strengths. We found that the yields of nucleohistone obtained from the mouse lymphoma by this method were somewhat low due to the fact that part of the nucleic acid present in the first molal NaCl extract did not precipitate on the sixfold dilution with water. Because of the possibility that this resulted from some degree of enzymatic depolymerization of the nucleate during the initial washing of the tissue, it was considered desirable to add an inhibitor of depolymerase to the solutions.[4] We therefore employed a procedure very similar to Petermann and Lamb's (*8*) modification of the method of Mirsky and Pollister. The details of our procedure follow.

All operations were carried out at 3° C. or less and the *p*H was always close to neutrality. The minced fresh tumor tissue was first homogenized into three volumes of 0.05 M sodium citrate (*p*H 7.1); a one-minute homogenization with the Waring blendor was adequate to break the cells and leave most of the nuclei intact. The nuclear components were separated by two successive centrifugations of the extract at 600 × g for 10 minutes. In preparations with frozen tumor tissue it was found that even a one-minute homogenization broke most of the nuclei, and centrifugation for 30 minutes at 1,400 × g was required to sediment all of the DNA-containing particulates. The sediments were joined, washed twice with the 0.05 M citrate, and then stirred for 48 hours with about 6 ml. of M NaCl-0.01 M citrate for each gram of original tissue. This extract was then centrifuged for 30 minutes at 40,000 × g and the clear supernatant was poured into six volumes of 0.01 M sodium citrate. The nucleohistone, which precipitated as long tight fibers, was collected on a stirring rod, washed with 0.14 M NaCl-0.01 M citrate, and redissolved in the M NaCl-0.01 M citrate. The cycle of precipitation and resolution was repeated three or four times and the final product was kept in M NaCl-0.01 M citrate. These solutions were in every case highly viscous and strongly doubly refractive.

Practically all of the DNA of the original tissue was found in the supernatant of the first M NaCl extraction, and essentially all of the DNA was precipitated on each 6-fold dilution of the solutions of nucleohistone in M NaCl. Most of the losses were due to difficulty in removing the last traces of the precipitate; no attempt was made to recover what could not be removed on the stirring rods. As shown in column 8 of table 1, the yields in four different preparations represented from 83 to

[4] In later work we have found that the major portion of the desoxyribonuclease activity of the lymphoma has a *p*H optimum near *p*H 5, is not activated by Mg^{++}, and is not inhibited by citrate. The lymphoma depolymerase thus resembles the enzymes described in the case of calf thymus and spleen by Maver and Greco (*7*). Mouse serum, however, contains a desoxyribonuclease which is activited by Mg^{++}, inhibited by citrate, and has an optimum at around *p*H 7. Extracts of the tumor always contained a small but measurable amount of such a *p*H 7 depolymerase activity; this is very probably derived from serum or intercellular fluids since it was not possible to free the tumor of these prior to homogenization. The added citrate inhibits the action of this *p*H 7 depolymerase rather than that of the tissue depolymerase itself. These results will be presented in detail in a later paper.

96 percent of the DNA of the tissue. The variation of the N/P ratio (column 9) among different preparations is about the same as the range of 3.9 to 3.4 for different preparations of beef spleen nucleohistone (*8*).[5]

Viscosity of Nucleohistone

The relative viscosity of four preparations of the nucleohistone (in M NaCl–0.001 M sodium citrate) as a function of concentration is given in text-figure 1. The viscosity was measured in an Ostwald-Fenske viscosimeter at 25° C.; the time for distilled water was 11.7 seconds. The values of viscosity were not affected by forced flow through the capillary, nor were they appreciably different after the solutions had stood at room temperature for several days. Although there was some variation among preparations, all of them exhibited a high viscosity which increased sharply with concentration and fell in the range reported for the nucleohistones of various other tissues (*6*, *8*). For comparison, the viscosity-concentration curve for our preparation of thymus nucleohistone which was used for studies of anomalous titration (*9*), is also given in text-figure 1.

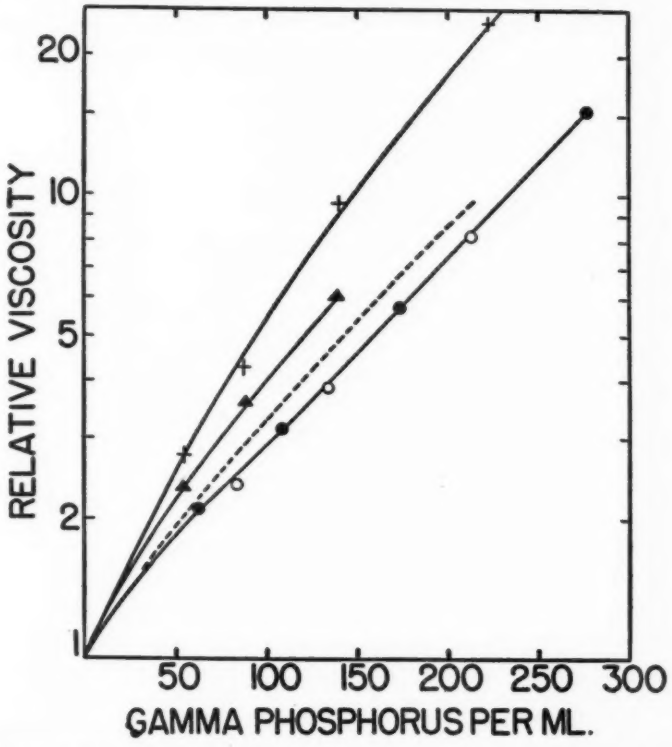
Attempts were also made to study the viscosity of the nucleohistone at low salt concentration. When the solution in M NaCl–0.01 M sodium citrate was dialyzed by stirring against 0.001 M sodium citrate, the nucleohistone at first precipitated as a fiber and then slowly redissolved just as do the nucleohistones of calf thymus (*6*) and beef spleen (*8*). No reliable values of the viscosity of such dialyzed preparations could be obtained. Although the initial viscosity was in every case considerably higher than that of the original solution in M NaCl, it gradually dropped upon standing at room temperature, reaching a value in 24 to 48 hours only slightly above that of water. Furthermore, the viscosity had a thixotropic character in that it was also lowered as a result of forced flow up and down the capillary of the viscosimeter. Addition of small amounts of salt to the freshly dialyzed solution likewise sharply lowered the viscosity; in a typical case the relative viscosity was reduced from 3.71 to 1.8 by the addition of 0.002 mEq. of NaCl per ml. to the solution of nucleohistone in 0.001 M sodium citrate.

When the freshly dialyzed solution is made 0.14 M in NaCl, a loose ribbonlike gelatinous fiber is formed which can be removed on a stirring rod. This easily redissolves in M NaCl to yield a solution with a stable viscosity that is close to that of the original solution of nucleohistone in M NaCl–0.001 M sodium citrate. When this is poured into 6 volumes of water the typical dense fiber is again formed. In this respect the change that occurs on removal of salt is reversible.

However, the change that occurs when the dialyzed solution stands at room temperature is apparently irreversible. Not only does the viscosity drop but the property of precipitating as a fiber in 0.14 M NaCl is lost, and on subsequent addition of NaCl to 1 M the viscosity remains low and no fiber formation occurs on sixfold dilution. The nature of the changes

[5] It is recognized that the DNA and histone are probably largely dissociated in molal salt and reassociated at very low salt concentrations, and that such reassociation does not necessarily restore in all respects the original relationship between the protein and nucleate (*cf. 6, 7, 9*).

that occur on standing at low salt concentration are unknown. Mirsky and Pollister (*6*) have suggested that certain irreversible changes occurring when thymus nucleohistone is exposed to a low salt concentration are due to enzymatic depolymerization, which is depressed in the presence of M salt. Our results are in harmony with this suggestion since the irreversible changes that occur at low salt concentration apparently result not from



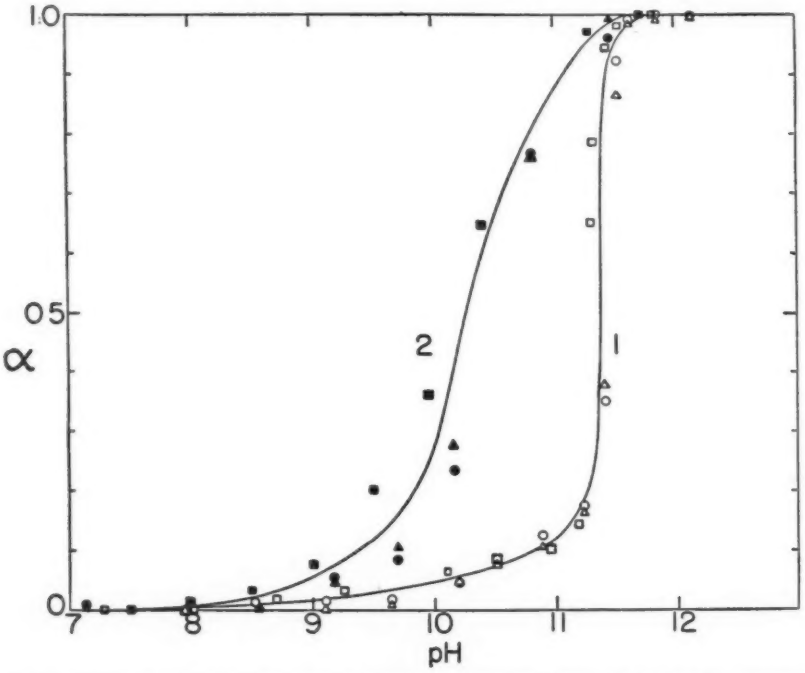
TEXT-FIGURE 1.—The viscosity of various preparations of the mouse lymphoma nucleohistone. ●, preparation 2; ○, preparation 3; ▲, preparation 5; +, preparation 7. The viscosity curve for a preparation of calf thymus nucleohistone (see text) is given by the dotted line.

the exposure to the low concentration of salt but from long standing under these conditions.

Anomalous Titration Behavior

Electrometric titration studies of a number of desoxypentose nucleic acids (*10-12*) have shown an anomalous titration behavior that appears to be one of the distinguishing characteristics of the native hydrogen-bonded macromolecular state of the nucleic acid. We have recently described (*9*) a spectrophotometric method for studying this anomalous titration that can also be used in the presence of protein, and it was

shown by this method that the anomalous titration behavior was also a property of the nucleic acid component of calf thymus nucleohistone prior to removal of the protein. It was considered of interest to determine whether the nucleic acid component of the mouse lymphoma nucleohistone exhibited the anomalous titration behavior and to what extent. The method used was exactly as described previously (*9*); the spectral changes on which the titrations of the lymphoma nucleohistone were based are similar to those given in figure 1 (inset) of that paper for calf thymus nucleohistone. The spectrophotometric titration curves of the mouse



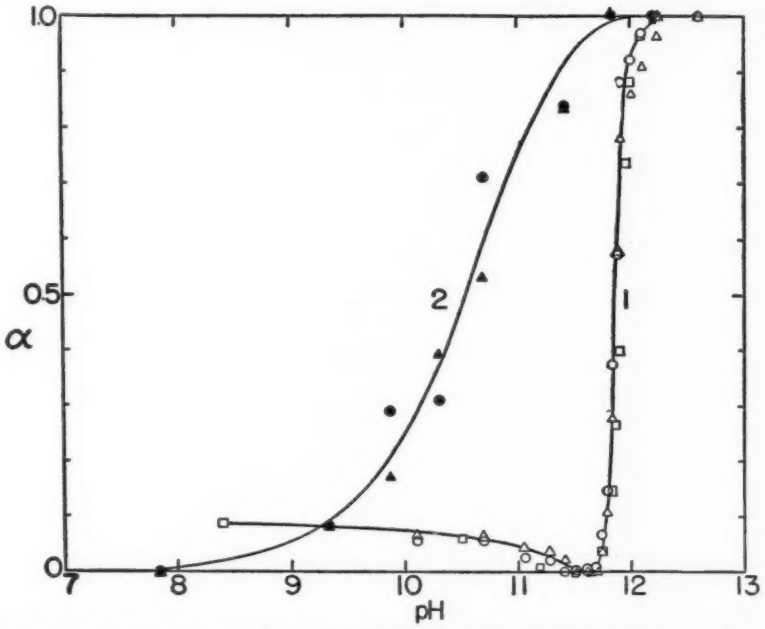
TEXT-FIGURE 2.—Spectrophotometric titration of nucleohistone in M NaCl. Curve 1, forward titration from neutrality to *p*H 12.15; Curve 2, back-titration from *p*H 12.15 to neutrality, ○ and ●, data for preparation 2 at 270 mμ; △ and ▲, at 230 mμ. □ and ■, data for preparation 5 at 230 mμ.

lymphoma in M NaCl and in water are given in text-figures 2 and 3 respectively. In each case data for two separate preparations are shown; results with other preparations were also in close agreement.

The results are quite similar to those previously found with calf thymus nucleohistone. Both text-figures 2 and 3 show the distinction between the abnormally steep and asymmetrical forward titration curves and the displaced, relatively symmetrical back-titration curves. In the case of the forward titration curve in water there occurs a slight initial decrease of optical density between *p*H 7 and 11.5, a leveling off, and then a very

sharp rise; α was set equal to zero at the constant value found between *p*H 11.5 and 11.7. The forward titration curve is seen to be considerably steeper in water than in M salt, but on addition of enough NaCl to the aqueous solution of nucleohistone to make the concentration 1 M, a curve identical with that shown in text-figure 2 is again obtained.

These results demonstrate that the anomalous titration behavior is a property of the DNA component of the mouse lymphoma nucleohistone. From the steepness and asymmetry of the forward titration curves, and the displacement of the back-titration curves, it appears that the degree of



TEXT-FIGURE 3.—Spectrophotometric titration of nucleohistone in water. Curve 1, forward titration from neutrality to *p*H 12.6; Curve 2, back-titration from *p*H 12.6 to neutrality. △ and ▲, data for preparation 2 at 230 mμ; ○ and ●, at 270 mμ. □, data for preparation 6 at 230 mμ.

hydrogen bonding in the mouse lymphoma DNA is approximately the same as in the calf thymus DNA.

Discussion

No comparison can be made at the present time between the nucleohistones of the lymphoma and of other tissues of the same species since the latter have not yet been isolated and characterized. With respect to the properties examined in this study, the lymphoma nucleohistone shows a behavior rather similar to that of various other nucleohistones that have been investigated. The fact that most of the DNA can be isolated from the lymphoma as the nucleohistone by a slight modification

of the salt method of Mirsky is in itself a point of resemblance. Similarities are also evident with respect to viscosity and solubility properties.

The finding of the anomalous spectrophotometric titration behavior with the lymphoma nucleohistone shows that its nucleic acid component is in an undegraded state and possesses a considerable degree of hydrogen bonding. These titration results also provide additional evidence for the view previously expressed that the blocking of the enolic groups through hydrogen bonding may be a universal property of undegraded DNA and that this blocking is not dependent on the process of removal of protein or precipitation of the nucleate (*9*). The data also demonstrate that, as with calf thymus nucleohistone, the anomalous titration effect is found whether the nucleate is largely dissociated from the histone, as in molal salt, or associated with the histone, as in low salt concentration.

Summary

The content of pentose nucleic acid and of desoxypentose nucleic acid of several generations of a transplantable mouse lymphoma has been determined. The tumor contains over three times as much desoxypentose nucleic acid as pentose nucleic acid.

The procedure is described by which most of the desoxypentose nucleic acid of the tumor can be isolated as the nucleohistone.

The nucleohistone has been characterized with respect to its viscosity, its solubility properties, and its anomalous spectrophotometric titration behavior. The results show that the nucleic acid component of the nucleohistone exists in an undegraded macromolecular state. The anomalous titration curves indicate that the degree of hydrogen bonding of the nucleic acid component is approximately the same as was previously found with the nucleic acid component of calf thymus nucleohistone.

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Desoxypentose Nucleic Acids and Nucleoproteins of Malignant Tissues. II. Physico-Chemical Studies of the Desoxypentose Nucleic Acid of a Transplantable Mouse Lymphoma [1]

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The first paper of this series (*1*) dealt with the isolation and partial physico-chemical characterization of the nucleohistone of the transplantable lymphoma L1 of the strain A mouse. This paper presents some physico-chemical studies of the highly polymerized desoxypentose nucleic acid that was prepared from this nucleohistone. It is largely concerned with a number of properites associated with the native hydrogen-bonded macromolecular state of the nucleate and its degradation by such denaturing agents as acid and alkali. These studies afford an opportunity to compare the physico-chemical behavior of the DNA[3] of a rapidly growing tumor with that of other DNA's that have been investigated by the same procedures. In addition, they provide the first extension of certain new techniques developed in this laboratory to a DNA other than that of calf thymus.

Materials and Methods

Two preparations (1 and 2) of highly polymerized mouse lymphoma DNA were made from the nucleohistones designated in the previous paper (*1*) as preparations 6 and 7. The protein was eliminated by 10 to 12 treatments with chloroform-octanol (8/1) (*2*) and the sodium nucleate was then precipitated in 60 percent ethanol, washed with 70 percent ethanol followed by absolute ethanol and ether, and dried in air. The material was in the form of white fluffy fibers. Both preparations were biuret-negative and contained less than one percent of pentose nucleic acid as determined by the phloroglucinol procedure (*3*); the N/P ratios were 1.78 and 1.75. The two preparations were closely similar with respect to the various physico-chemical properties with which this paper is concerned.

[1] Received for publication November 19, 1952.

[2] National Institutes of Health, Public Health Service.

[3] In order to conserve space, DNA is used as an abbreviation for desoxypentose nucleic acid. MLDNA and CTDNA will refer to the DNA of the transplantable mouse lymphoma and of calf thymus respectively.

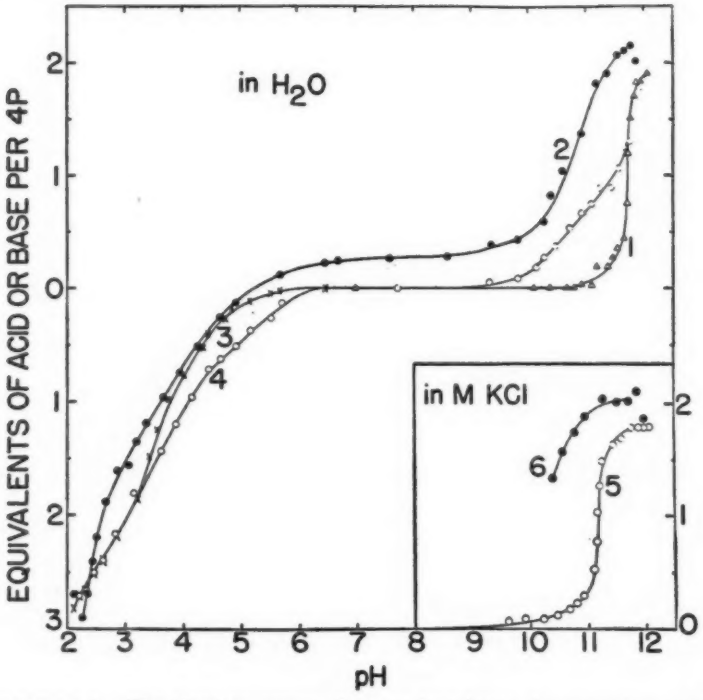
Alkali-treated nucleate was made by bringing a solution of the nucleate to a *p*H of 12.4, reneutralizing after 10 to 20 minutes, and dialyzing free of salt.

The methods used in this investigation have been described in detail elsewhere and reference to these descriptions will be made in each case.

Results

Electrometric Titration

Recent work on the electrometric titration of several desoxypentose nucleates indicates that one of the distinguishing characteristics of the undenatured macromolecular state of DNA is the anomalous titration behavior (*4-8*). In view of this we have carried out electrometric titrations of MLDNA as an important aspect of its physico-chemical characterization. The *p*H was measured with glass electrodes that were calibrated as described previously (*8*); corrections for free acid and base were estimated from titrations of water or appropriate salt solutions. The curves



TEXT-FIGURE 1.—The titration curves of mouse lymphoma desoxypentose nucleate. Curve 1, △, titration with alkali from *p*H 7.0 to *p*H 12.1. Curve 2, ●, back-titration with acid from *p*H 12.1. Curve 3, ×, titration with acid from *p*H 7 to *p*H 2.1. Curve 4, ○, back-titration with alkali from *p*H 2.1. Curve 5, ○, titration in M KCl with alkali from *p*H 7 to *p*H 12.1. Curve 6, ●, back-titration in M KCl with acid from *p*H 12.1. Concentration of nucleate, 0.463 percent. Temperature, 26.5° C.

in text-figure 1 show that the anomalous titration behavior is also found with MLDNA. These curves resemble those given by Lee and Peacocke (7) in that the back-titration curves from the acid and alkaline side (curves 2 and 4) are significantly different from each other. Possible explanations for this difference were discussed in detail by Lee and Peacocke (7), and our results appear to be in harmony with their conclusions.

A titration in M KCl is given in the inset; the back-titration in this medium could not be carried below *p*H 10.4 because of the beginning of precipitation [*see also* (4)]. The finding of the anomalous titration behavior in M salt is in agreement with the conclusion of Gulland *et al.* (4) that the decrease of viscosity caused by electrolytes does not involve rupture of the hydrogen-bonded structure.

The titration curves show close to 1.85 groups per 4 P titrating between *p*H 7 and 12.2, and approximately 2.9 groups per 4 P titrating between *p*H 7 and 2.2. In the absence of data on the content of purine and pyrimidine residues no analysis of the curves in terms of titration constants is reported at the present time.

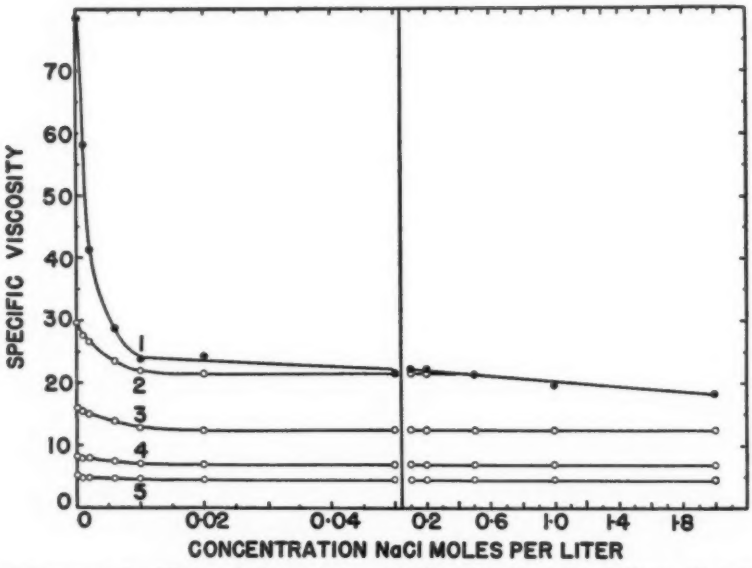
Viscosity

The high anomalous viscosity of DNA has been the subject of considerable study. The striking effects of electrolytes (*9–11*) and of acid and alkali (*9, 11–14*) in reducing the viscosity of solutions of DNA provide important information with respect to its physico-chemical state. Solutions of both preparations of MLDNA possessed high viscosities which increased sharply with concentration and showed marked dependence on the applied pressure.

The influence on the viscosity of the addition of sodium chloride to an aqueous solution of MLDNA is given in text-figure 2. In order to facilitate future comparisons with data on other nucleates it was considered desirable [*see also (10)*] to relate the viscosity to the salt concentration at a constant velocity gradient rather than at constant applied pressure. To do this the viscosity was determined at each salt concentration at a number of different applied pressures and from these data a series of curves relating the viscosity to velocity gradient at each salt concentration were constructed. From these could be estimated the viscosity for each salt concentration at a given velocity gradient. The curves relating viscosity to salt concentration at 4 different velocity gradients are given in text-figure 2; the data obtained under gravity are also plotted. It is to be noted from text-figure 2 that the higher the velocity gradient, the less is the effect of salt on viscosity, and that the variation of viscosity with the velocity gradient is much larger the lower the salt concentration (at values below the critical concentration of about 0.01 M) [*see also (10)*].

The effects of acid and alkali in lowering the viscosity of solutions of DNA has been correlated with the destruction of the original hydrogen-bonded structure by acid or alkali (*4, 11*). We considered it desirable to establish the viscosity–*p*H relationship for the tumor DNA, but for

purposes of future comparisons it was again considered advisable to relate the viscosity to *p*H not at constant applied pressure but at constant velocity gradient. The solution of DNA was carefully titrated to the desired *p*H and the viscosity determined at a number of different applied pressures. Curves relating the viscosity to the calculated velocity gradient at each *p*H were used to construct the curves shown in text-figure 3, relating the viscosity to *p*H at fixed velocity gradients. It is



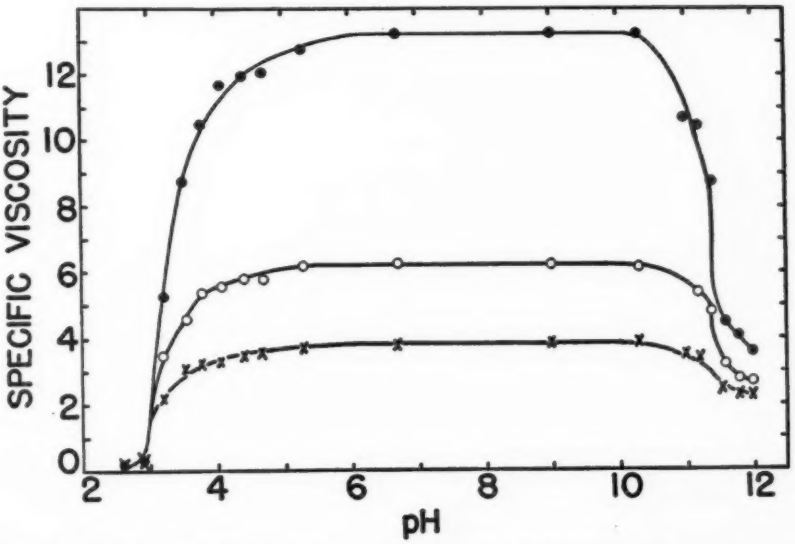
TEXT-FIGURE 2.—The variation of the specific viscosity of solutions of mouse lymphoma desoxypentose nucleate with concentration of added sodium chloride. Curve 1, under gravity. Curves 2 to 5, at constant velocity gradients of 120, 289, 867, and 2,020 sec.$^{-1}$ respectively. Concentration of nucleate, 0.31 percent. Temperature, 26.3° C.

seen that the viscosity remains constant between about *p*H 5.5 and 10.5, and these critical limits are seen to correspond fairly closely with the beginning of titration (curves 1 and 3 of text-fig. 1). The reduction of viscosity with *p*H is seen to be greater the lower the velocity gradient, this being a reflection of the greater dependence of the viscosity on the velocity gradient in the constant region between *p*H 5.5 and 10.5 than at the extremes of *p*H.

Absorption Spectra

It has been shown (*8*, *15*) that the ultraviolet absorption spectrum of a solution of calf thymus DNA (CTDNA) depends on the *p*H, the type and concentration of salt present, and the previous treatment of the DNA. Since the curves relating the absorption to *p*H and to salt concentration yield significant information regarding structure and macromolecular state, it was considered desirable to extend the studies to mouse lymphoma

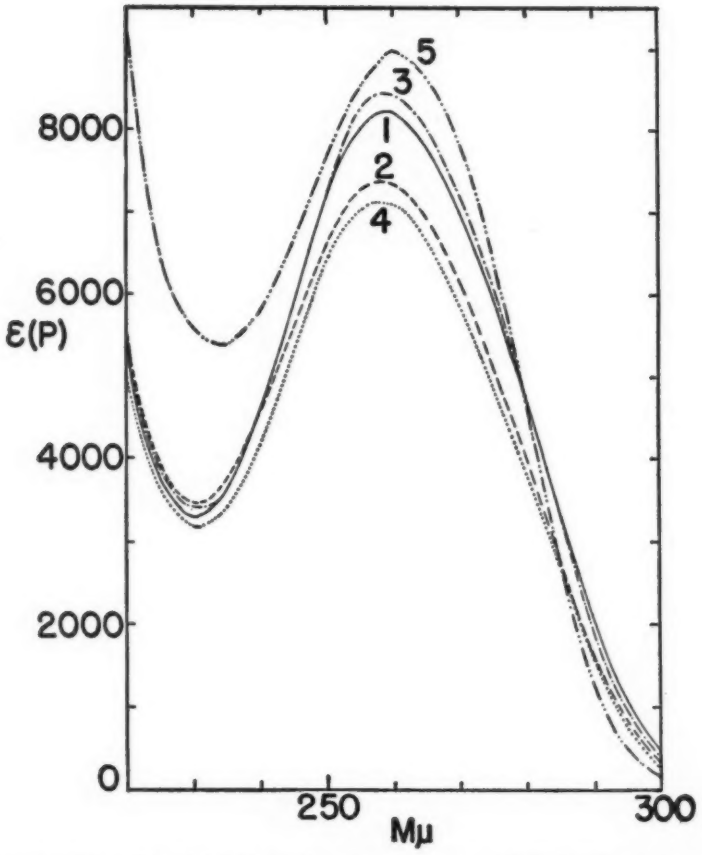
DNA (MLDNA). The influence of NaCl and alkali on the ultraviolet absorption spectra are shown by the curves of text-figure 4. Text-figure 5 shows the dependence of the optical density (at 260 mμ) of solutions of the native and of alkali-treated DNA on the total concentration of added NaCl or $MgCl_2$. It is evident: 1) that the addition of salts at neutrality causes a lowering of absorption in the region of the maximum, the extent of lowering increasing with the quantity of added salt up to a critical concentration beyond which the absorption remains constant; 2) that far less of $MgCl_2$ than of NaCl is required to bring about a given lowering of absorption; 3) that the quantity of Mg^{++} required to cause the transformation is of the same order of magnitude as the quantity of DNA P



TEXT-FIGURE 3.—The variation of the specific viscosity of solutions of mouse lymphoma desoxypentose nucleate with *p*H. Concentration of nucleate, 0.282 percent. Solutions initially 0.05 M in NaCl. Temperature, 26.1° C. The velocity gradients were ●, 289 sec.$^{-1}$; ○, 867 sec.$^{-1}$; ×, 2,020 sec.$^{-1}$.

present; 4) that the curves with the native DNA, but not with the alkali-treated DNA, are two step in character; and 5) that more NaCl is required to bring about the spectral change with alkali-treated DNA than with native DNA.

Similar data were obtained with a variety of other salts. The curves relating D/D_0 to concentration that were obtained with KCl, Na_2SO_4, and sodium citrate were close to those shown for NaCl, while the curves for $MgSO_4$, $SrCl_2$, $BaCl_2$, and $CaCl_2$ were almost the same as those given for $MgCl_2$. The ultraviolet absorption spectra obtained in the presence of enough salt to cause the maximum change were similar for all of the salts with both the original and alkali-treated DNA. These results are in harmony with the view expressed elsewhere (*15*) that these spectral



TEXT-FIGURE 4.—Ultraviolet absorption curves of mouse lymphoma desoxypentose nucleate. ε (P), atomic extinction coefficient with respect to phosphorus. Curve 1, original nucleate in water. Curve 2, original nucleate in M NaCl. Curve 3, alkali-treated nucleate in water. Curve 4, alkali-treated nucleate in M NaCl. Curve 5, nucleate in 0.02 M NaOH or in 0.02 M NaOH–M NaCl.

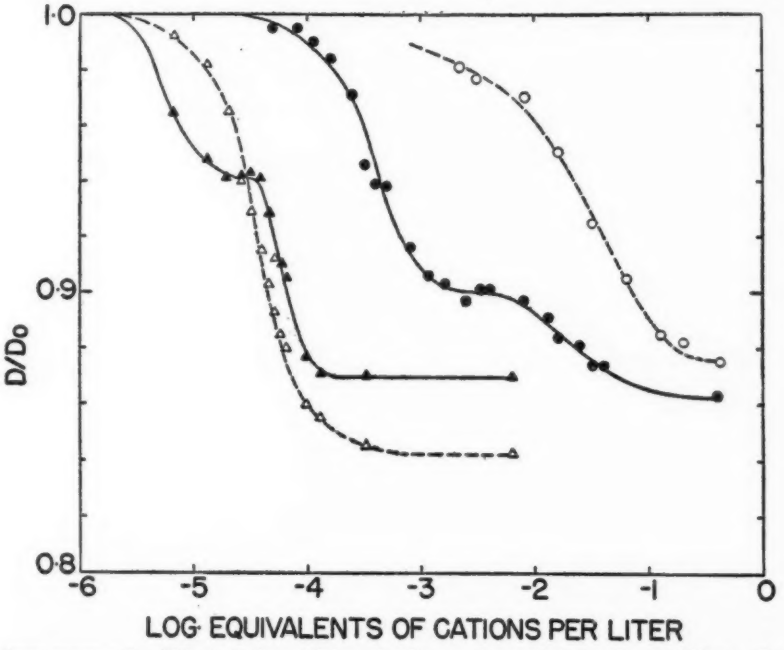
changes are associated with binding of cations, and that the divalent cations are very tightly bound by the DNA.

Spectrophotometric Titrations

A recent paper from this laboratory (*8*) described a spectrophotometric method for study of the anomalous titration phenomenon which depends on an analysis of the changes of ultraviolet absorption of DNA with *p*H in the *p*H region 7 to 12.5. The use of this method with CTDNA (*8*) and the nucleohistones of calf thymus (*8*) and mouse lymphoma (*1*) has been reported. A study was also made of the mouse lymphoma DNA and the results of this are given below. The method was exactly as described

previously (*8*). However, knowledge of the influence of salts on the absorption of DNA [(*15*), *see also* text-figures 4 and 5 of this paper] now enables a more critical choice to be made of the best conditions for determination of the spectrophotometric titration curves.

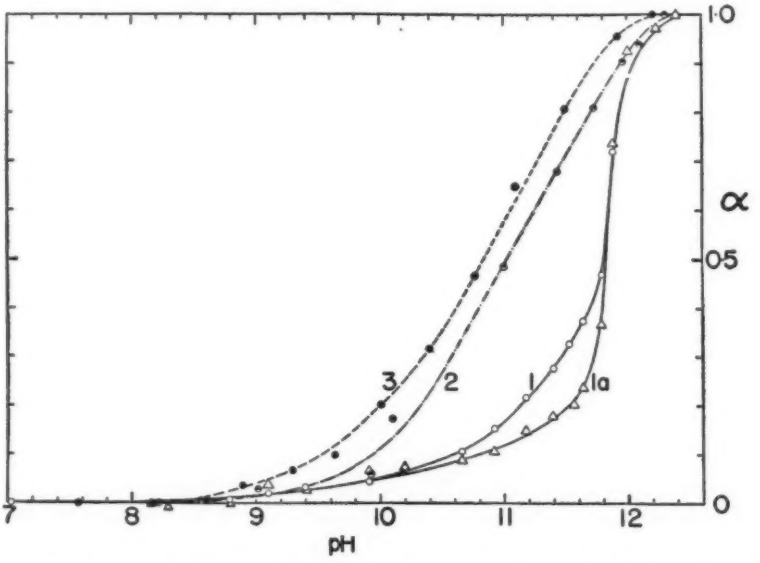
It is seen from the curves of text-figure 4 [*see also* figures 2 and 4 of (*15*)] that the optical density at 230 mμ of DNA, either native or degraded, is



TEXT-FIGURE 5.—Effect of the addition of salts on the optical density at 260 mμ of a solution of mouse lymphoma desoxypentose nucleate in water. The ordinate is given as the ratio of D, the optical density in the presence of a given concentration of salt, to D_0, the optical density in the absence of added salt. ▲, addition of $MgCl_2$ to untreated nucleate. △, addition of $MgCl_2$ to alkali-treated nucleate. ●, addition of NaCl to untreated nucleate. ○, addition of NaCl to alkali-treated nucleate.

practically independent of the salt concentration and is primarily determined by the *p*H between *p*H 7 and 12.2. Consequently, the changes of salt concentration that occur as a result of the addition of acid or alkali have no effect on the spectral titration curves determined at this wave length except as a result of their influence on activity coefficients. By contrast, the optical density at 260 mμ of both native and degraded DNA is strongly dependent on the concentration of salt in the neutral region (but not beyond *p*H 12). As a result, the change of absorption at 260 mμ that occurs on addition of alkali represents a resultant of an initial lowering due to increase of salt and a rise at higher *p*H associated with the titration of the enolic hydroxyl groups. This is illustrated by the titration shown in text-figure 6, which was carried out in the presence of 0.006 M boric

acid. The initial density at 260 mμ was 0.778. On adding alkali this dropped with each increment up to a *p*H of 8.15, at which it was 0.692. On further addition of alkali the optical density remained constant until *p*H values beyond 8.8. The level portion, from *p*H 8.15 to 8.8, corresponds to concentrations of sodium borate of from 6×10^{-4} to 2.5×10^{-3} (log Na^+ from -3.2 to -2.6); this range is seen to fall on the first plateau of curve 1 of text-figure 5, a region where the optical density changes very little with concentration of salt. In the analyses of the data at 260 mμ, α was set equal to zero at the constant value of optical density found between *p*H 8.15 and 8.8. When NaCl to a concentration of 0.001 or greater is added to the solution of nucleate prior to titration, no further



TEXT-FIGURE 6.—Spectrophotometric titrations of mouse lymphoma desoxypentose nucleate in water containing 0.006 M boric acid. Curves 1 and 1a, forward titration curves from data at 230 and 260 mμ respectively. Curve 3, back-titration from *p*H 12.4, at 230 mμ. Curve 2, subsequent titration from neutrality to *p*H 12.4, at 230 mμ. $\alpha = D - D_1/D_2 - D_1$, D being the optical density at a given *p*H and D_1 and D_2 the optical densities for $\alpha = 0$ and 1.0 as estimated from the asymptotes of the curves.

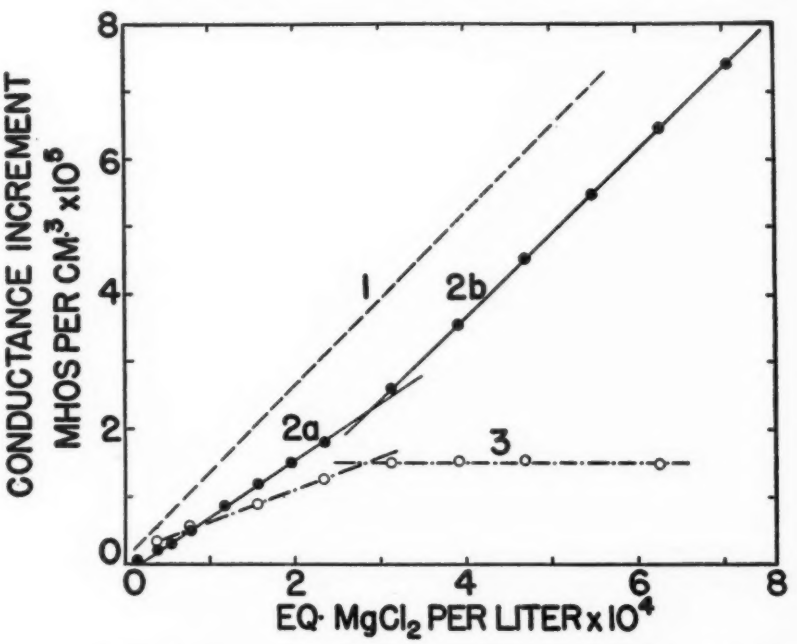
drop occurs with the first increment of alkali, and the spectrophotometric titration curve from data at 260 mμ is essentially the same as curve 1a of text-figure 6.

The spectrophotometric titration curves of MLDNA given in text-figure 6 are similar to, although not identical with, those previously found for CTDNA (*8*). As with the CTDNA (*8*), a similar shift of the spectrophotometric titration curve is observed on exposure of the MLDNA to acid or to heat. These results afford additional evidence of the existence of a considerable degree of hydrogen bonding in MLDNA.

Studies on Ion Binding

Results obtained from dialysis equilibrium experiments (*16*, *17*) with calf thymus DNA show that at neutral *p*H considerable binding occurs between sodium ions and the phosphate residues of DNA, and as a result the negative charge borne by the nucleate in solution of sodium salts is considerably less than 4 per 4 atoms P. We have carried out similar experiments with MLDNA by the procedure described earlier (*17*); the values of negative charge per 4 P atoms are 1.4 in 0.005 M NaCl and 2.11 at 0.05 M NaCl. These results are of the same order of magnitude as those previously found with our preparation of CTDNA (*17*) but show a greater trend toward higher values of charge with increasing ionic strength. They indicate that considerable binding (*ca.* 1.9 to 2.6 atoms Na per 4 P) occurs between Na^+ and MLDNA.

The curves relating ultraviolet absorption to concentration of salt were interpreted to indicate that divalent cations are far more tightly bound by DNA than are univalent cations. This was confirmed in the case of CTDNA by a conductometric procedure (*15*). Similar experiments have been carried out with MLDNA and salts of sodium and of divalent cations. The method was exactly as described elsewhere (*15*). Results were fairly



TEXT-FIGURE 7.—Conductance increment curves for addition of $MgCl_2$ to a solution of mouse lymphoma desoxypentose nucleate. Curve 1 represents the increase of specific conductance that occurs on addition of $MgCl_2$ to water, and Curve 2 that on addition of $MgCl_2$ to a solution of the nucleate. Curve 3 gives the difference between Curves 1 and 2. Nucleic acid phosphorus, 3.74×10^{-4} M.

similar to those obtained with the CTDNA. The increase of conductance that occurs on adding NaCl or Na_2SO_4 to a solution of MLDNA is very close to that found on adding the salts to water. By contrast, a 2-phase conductance increment curve with a lowered initial slope is obtained on addition of the salts of divalent cations to the solution of MLDNA. A typical experiment with $MgCl_2$ is shown in text-figure 7, which shows the lowered initial slope (line 2a) and the close approximation to the water curve of line 2b after addition of 0.79 equivalents of Mg^{++} per atom of DNA P. Data with other salts, at different concentrations of DNA and with preparation 2 are summarized in table 1. The slopes of the water curves are 0.127, 0.141, and 0.135 for $MgCl_2$, $BaCl_2$, and $CaCl_2$, respectively. The values of the intercepts are seen to be quite close for Mg^{++}, Ca^{++}, and Ba^{++}. The constancy of the intercept (in equivalents of Mg^{++} per atom of DNA P) at different concentrations of DNA provides evidence of a tight and almost complete binding of the added Mg^{++} by the MLDNA [also discussed in (*15*)].

TABLE 1.—*Conductance increment data for mouse lymphoma desoxypentose nucleic acid and divalent cations*

	Salt	Concentration of DNA P	Intercept	Slope of line a	Slope of line b
		M. per l.$\times 10^4$	*equivalents divalent cation per atom of DNA P*	*mhos per cc. per equivalent of ion per l.*	*mhos per cc. per equivalent of ion per l.*
Prep. 1	$MgCl_2$	1. 78	0. 73	0. 087	0. 127
	$MgCl_2$	3. 74	0. 79	0. 083	0. 125
	$MgCl_2$	7. 14	0. 76	0. 093	0. 123
	$CaCl_2$	3. 74	0. 78	0. 084	0. 129
	$BaCl_2$	3. 74	0. 81	0. 086	0. 136
Prep. 2	$MgCl_2$	7. 5	0. 75	0. 088	0. 122
	$CaCl_2$	7. 5	0. 81	0. 091	0. 133
	$BaCl_2$	7. 5	0. 74	0. 087	0. 132

Discussion

The electrometric and spectrophotometric titration curves and the viscosity-*p*H curves show that the MLDNA possesses a considerable degree of hydrogen bonding, and that its initial hydrogen-bonded macromolecular structure is degraded by exposure to high or low *p*H. The existence of strong interaction of the MLDNA with ions is demonstrated by curves relating viscosity, ultraviolet absorption, and conductivity to the quantity of added salt. In all these respects the MLDNA resembles other DNA's that have been isolated by similar mild methods. The only other DNA that has thus far been investigated by all the procedures used here is our preparation of CTDNA. Comparison of the data of this paper with that presented elsewhere for the CTDNA (*8*, *15*) shows that although the general disposition of the various curves are somewhat similar, they are by no means identical. For example, the viscosity of the two preparations of MLDNA were within a few percent of each other but

only 60 percent of that of the CTDNA, and the forward spectrophotometric titration curves of the MLDNA are less steep than those of the CTDNA. This comparison will not be carried further at present since the two nucleates, derived from different species, are unquestionably not the same. The more significant comparison from the point of view of oncology, that between the MLDNA and DNA of normal tissues of the same animal, cannot be made at present since the latter have not yet been isolated and characterized.

Summary

The desoxypentose nucleic acid of the transplantable lymphoma L1 has been characterized with respect to a variety of physico-chemical properties. The studies included: 1) electrometric titration of the acidic and basic groups; 2) determination of the influence of electrolytes and of acids and alkali on the viscosity; 3) determination of the ultraviolet absorption spectra of the native and alkali-treated nucleate and of the variation of these spectra with *p*H and with concentration and type of added salt; and 4) estimation of the binding of the nucleate with ions by means of dialysis equilibrium and conductometric procedures.

The results demonstrate that the lymphoma nucleate possesses a hydrogen-bonded macromolecular structure analagous to that found previously with other desoxypentose nucleates. The behavior of the lymphoma nucleate, with respect to the properties examined in this investigation, is somewhat similar to that of the desoxypentose nucleate of calf thymus, the only other nucleate that has thus far been studied by all of the methods listed above.

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Protecting Action of Citrate on the Agent of Chicken Tumor I (Rous Sarcoma Virus) During Roentgen Radiation *in Vitro*[1]

W. Ray Bryan, Egon Lorenz, *and* Dorothy Calnan,[2] *National Cancer Institute,*[3] *Bethesda, Md.*

In previous studies (*1*) on the effects of X radiation on the agent of chicken tumor I [Rous sarcoma virus (*2*)] *in vitro*, normal rabbit serum was added to the suspending media for the purpose of "protecting" the agent against indirect effects of the irradiation [*cf.* Lea (*3*)]. Preparatory to further investigations designed to include the indirect effects (*i.e.* effects in the absence of serum, or other known protecting agent), preliminary tests were carried out to determine whether citrate buffers, in which the agent was known to remain stable (*4*, *5*), would be suitable as suspending media for such studies. It was found that 0.025 M potassium citrate protected the agent to a considerable extent, and it seemed apparent that citrate buffers could not be used for the purpose stated. However, the protecting action of citrate was, in itself, of sufficient interest to merit further investigation, and additional experiments to confirm and extend this observation were performed. The experiments and results are described herein.

Materials and Methods

Irradiation of tumor agent suspensions.—The X-ray machine and the procedures employed were the same as previously described (*1*). Irradiations were carried out at room temperature, which did not vary more than 1° or 2° C. during the course of an experiment. The two, opposed X-ray tubes were operated at 186 kv. peak and 20 m. amps. Except for the inherent filtration of tube wall and bakelite window, which is equivalent to approximately 0.2 mm. of copper, no additional filtration was used. The effective wave length of the X radiation was of the order of 0.2 Å. The dose rates for different experiments varied from 4,500 r per minute to 4,700 r per minute, and the times of irradiation were adjusted so that total doses of either 0.7×10^5 r or 1.4×10^5 r were delivered. The dose-rate measurements were done in a rice phantom and were estimated to have an experimental error of not more than ± 10 percent. The dose rate obtained in the phantom agreed with that found in air within the limits of error.

Bioassays.—The standard of reference ("known") used to establish the 100 percent potency level in the assay of each irradiated suspension ("unknown") was a sample of a control suspension taken at the same time, and identical with the unknown suspension in every respect except that of irradiation.

[1] Received for publication January 21, 1953.

[2] The authors are indebted to Mr. Carl L. White and Mrs. Marguerite T. Wood for statistical analyses of the data included herein, and to Mr. Jerome Cornfield for valuable advice concerning the methods of analysis.

[3] National Institutes of Health, Public Health Service.

The bioassay procedure involved the use of the latent period response and a balanced experimental design for control of the biological variables (*6*). In most of the experiments some of the materials showed decreases in potency of more than a factor of 100 and the design and conditions of the bioassays were not entirely satisfactory. Many of the test chickens died from tumors induced by the stronger (control) inoculums before the weaker (unknown) inoculums had had full opportunity to produce their effects [see reference (*6*)]. Special statistical procedures [4] were used for the recovery of balanced information and the estimation of potency in these instances, but the experimental errors associated with the potency estimates were not readily determinable.[5] For this reason, and because of the large amount of computational labor involved, the experimental errors were not determined for the remaining potency estimates of these experiments. Since the procedures and the numbers of test chickens (36 to 40 per bioassay) were essentially the same as those previously employed (*1*), it is assumed that the standard errors of the log percent potency estimates, which did not differ from their reference standards (*i.e.* 2.0 log percent) by more than 2 $\log_{10}$ intervals, will be comparable to those previously found and that they fall within the limits of about ± 0.2 and ± 0.4 $\log_{10}$ intervals, with an average value of about ± 0.3.

Preparation of tumor agent suspensions.—The method of preparation of the partially purified tumor agent suspensions was the same in all experiments. It was selected for use in these studies not because it was considered an efficient purification method in terms of total yield, but because it had been found in the course of other investigations [6] to give freely dispersed suspensions of microsome particulates within the diameter range 30 mμ to 100 mμ which were relatively free of aggregated material (as determined by electron microscopy [6]). The latter is known to complicate the interpretation of irradiation data [see reference (*1*)]. At the time these studies were begun the method described below was the most efficient that had been found for producing this type of biologically active suspension. The details of the procedure were as follows:

Previously frozen tumor tissue was homogenized (by means of a Waring blendor) in 0.025 M sodium citrate solution in an amount that gave a 6.6 percent suspension of the tumor tissue. The suspension was clarified by ordinary centrifugation and the supernatant fluid was recovered and diluted with an equal volume of 1.4 M sodium citrate solution. The resulting suspension, in approximately 0.7 M sodium citrate, was allowed to stand for about 30 minutes [during which time aggregates of precipitated material formed—*cf.* reference (*5*)] and centrifuged [7] at 10,000 $\times$ g for 1 hour to sediment the aggregated fraction as well as any larger subcellular particulates that may have remained after ordinary centrifugation. The middle fraction of the supernatant fluid was carefully removed without disturbing the sediment or the top (centripetal) layer of the supernatant. The recovered fraction was then centrifuged at 30,000 $\times$ g for 2 hours, to sediment the remaining, smaller, microsome constituents. The pellets of the latter sedimented material were recovered and resuspended in 0.025 M sodium citrate of an amount equal to $\frac{1}{10}$ the original volume of the homogenate processed. The pellet material was thoroughly dispersed by blending for 2 minutes in a Waring blendor, and the suspension was clarified by centrifugation [7] at 10,000 $\times$ g for 5 minutes. The supernatant fluid was recovered and carried through a second, and final, cycle of alternate high-speed (30,000 $\times$ g; 1 hour) and low-speed (10,000 $\times$ g; 5 minutes) centrifugation.[8] The pellets sedimented during the last high-speed run were portioned among, and resuspended in, equal lots of the various reagents involved in a given experiment, before being clarified by the final low-speed

[4] Devised by Mr. Jerome Cornfield.

[5] Further development of the special statistical procedures (see footnote 4) did not seem justified because the experimental design employed [*i.e.* the 4 $\times$ 4 Latin square (*6*)] was obviously not efficient for bioassays of this agent involving large potency differences (*e.g.* several powers of 10). A more satisfactory method for this purpose, involving a paired wing site design, will be described in a subsequent report.

[6] Joint studies with Dr. H. Kahler, in progress.

[7] Model L Spinco ultracentrifuge, No. 20 head, manufactured by the Specialized Instruments Corporation, Belmont, California.

[8] No. 40 head of a Spinco ultracentrifuge. See footnote 7.

run. The volume of reagent used in each case was kept relatively the same as had been used in the preceding cycle (*i.e.* $\frac{1}{10}$ the original volume of tumor homogenate processed). The agent suspensions were kept cold (0°–8° C.) during preparation, and at all other times except during the process of irradiation, when they were handled at room temperature (see above).

Reagents.—The water used as suspending medium and in the preparation of all materials and reagents was triple distilled in glass equipment.

The citrate compound used in the final suspending media in all experiments was the tripotassium salt of citric acid, containing 1 water of crystallization.

Experiments

Five experiments involving as many different batches of partially purified tumor agent were carried out at different times over a period of seven months. The experimental procedures were as follows:

Experiment 1.—Suspensions of tumor agent in 0.025 M potassium citrate solution, in distilled water, and in 0.025 M potassium citrate solution containing 2 percent normal horse serum, were irradiated at a dose rate of 4,700 r per minute. Samples of each were taken for bioassay after 15 minutes of irradiation (total dose$=0.7 \times 10^5$ r) and after 30 minutes of irradiation (total dose$=1.4 \times 10^5$ r). As each sample was taken, a subsample of it was diluted immediately (within 1 minute) with citrate buffer (*p*H 6.7; 0.05 M) and inoculated into test chickens along with a paired subsample of a control agent suspension taken at the same time (see section on Bioassays). After this initial testing the materials were placed in an ordinary refrigerator (3°–8° C.) and were again tested for biological activity 4 hours later. The immediate and 4-hour tests are designated as parts *a* and *b* respectively, of experiment 1.

Experiment 2.—Suspensions of tumor agent in 3 different molar concentrations of potassium citrate solution (1.6, 0.16, and 0.016), in distilled water, and in 0.016 M potassium citrate solution containing 2 percent normal horse serum, were given a total dose of 0.7×10^5 r of X radiation. Samples were taken for bioassay, *a*) immediately after irradiation (within 1 minute), *b*) after standing in a refrigerator (3°–8° C.) for 4 hours following irradiation, and *c*) after standing for 24 hours under the same condition.

Experiment 3.—Experiment 3 was similar to experiment 2 except that it involved a different dosage of X radiation, namely 1.4×10^5 r, and only 2 different concentrations of citrate (0.16 M and 0.016 M). The serum-citrate suspension was exactly the same as in experiment 2. In addition to the bioassay of each irradiated suspension against its respective control, a bioassay was also carried out at each time interval in which the distilled water control (which had been found to be unstable in experiment 2, as indicated by the primary chicken responses) was compared directly with a simultaneous sample of the stable serum-citrate control.

Experiment 4.—Experiment 4 involved a constant dosage of X radiation, namely 0.7×10^5 r, and serial 10-fold concentrations of potassium citrate from 1.6×10^{-2} M through 1.6×10^{-5} M. Suspensions of tumor agent in the various concentrations of citrate, and in distilled water, were given the constant dose of irradiation and samples were taken immediately for bioassay.

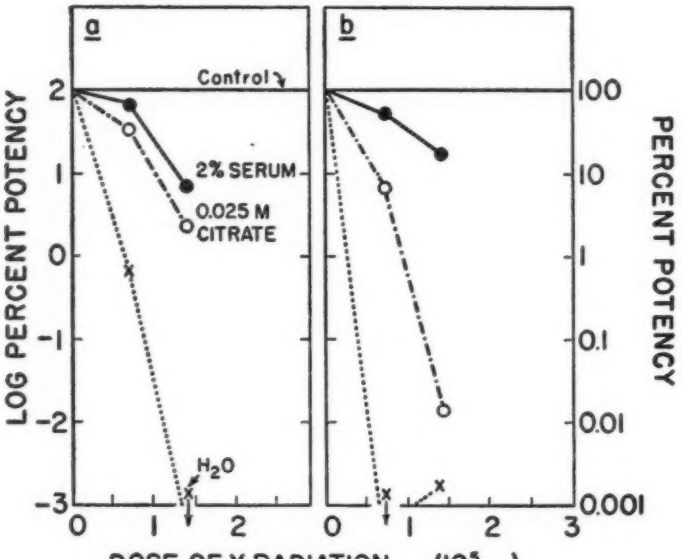
Since preparation of the tumor agent involved processing it in strong citrate solution (see section on Materials and Methods), the "distilled water" suspension could be expected to contain traces of citrate. Extreme care was therefore taken in resuspending the pellets of sedimented material in this experiment to prevent the carrying over of any more than necessary of the citrate adherent to the walls of the centrifuge tubes. After the tubes had been allowed to drain thoroughly on blotting paper, they were held at an angle and only enough suspending fluid was added to cover the pellets of sedimented material and permit their rough resuspension and transfer to other tubes. From the relative areas of the tubes with which the fluid came in contact, the measured quantity of fluid required to wet the entire tube surfaces, and the concentrations of citrate involved in the various purification steps, it was estimated that the residual

citrate in the final "distilled water" suspension might be expected to fall within the limits 10^{-6} M and 10^{-5} M. The results were therefore considered as representing a citrate concentration of $10^{-5.5}$ M, the logarithmic midpoint between these limits (This estimate does not apply to the "distilled water" suspensions of the other experiments since the same precautions were not observed therein during preparation and no basis for estimating the "trace" amounts of citrate was available in these instances.)

Experiment 5.—In the fifth and final experiment of this series, samples of suspending media of the various types were irradiated and then added to active tumor agent preparations. Twenty-milliliter lots of 0.16 M and 0.016 M potassium citrate, of distilled water, and of 0.016 M potassium citrate containing 2 percent normal horse serum, were irradiated with total doses of 1.4×10^5 r of X radiation. An aliquot of each, taken immediately after irradiation, was then added to an equal quantity of tumor agent suspended in medium of the same type, but nonirradiated. The control agent suspensions were diluted in like manner with equal quantities of the respective nonirradiated suspending media. Samples of the mixtures were taken for bioassay *a*) after 3 minutes, *b*) after 3 hours, and *c*) after 24 hours, the materials being kept in a refrigerator (3°–8° C.) during the periods between samplings. As in experiment 3, the distilled water control was bioassayed at the successive time intervals, against the serum-citrate control.

Results

The results are presented in a series of text-figures. The plotted points represent estimates of residual biological activity in (A) irradiated tumor agent suspensions (see text-figs. 1–6), or (B) suspensions of tumor agent in previously irradiated suspending media (text-fig. 7). In addition to the primary purposes of the experiments, certain of the data permitted analyses to be made (C) with respect to the nature of the irradiation dose-sur-



TEXT-FIGURE 1.—Data of experiment 1. Residual tumor-producing potencies of suspensions of tumor agent in different suspending solutions after irradiation.

a. Immediately after irradiation.

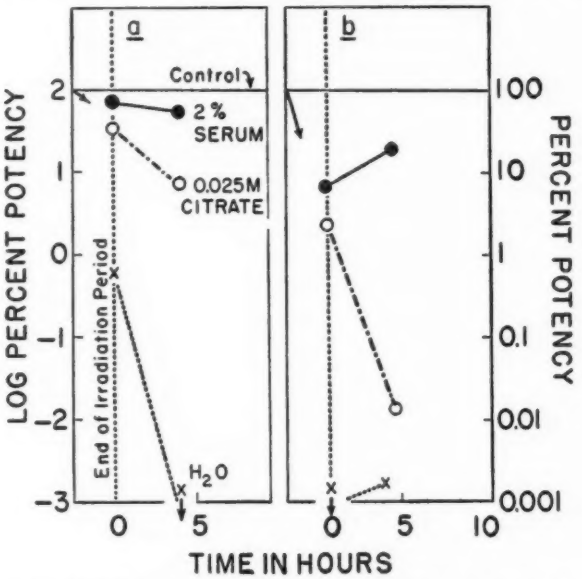
b. After standing for 4 hours at refrigerator temperature (3°–8° C.).

vival curve for direct effects (see text-figs. 8 and 9) along the lines employed in a previous report (*1*). The control line drawn at 2.0 log percent (100 percent) potency in each figure represents the assigned potency value for any given paired-control sample ("known") used as reference for bioassay of the irradiated sample ("unknown")—see section on Bioassay.

The suspensions of tumor agent in *a*) distilled water, and in *b*) citrate solution containing 2 percent normal horse serum, served as general controls for judging the relative protecting action of citrate. The former *a*) represent the effect of X radiation on the agent in the absence of competing, or "protecting," substances; the latter *b*) show the direct effects of the irradiation in the presence of a substance (serum) which, at the concentration used, is known to give maximal protection against the indirect effects [*cf.* Lea (*3*) and reference (*1*)].

A. Irradiated Tumor Agent Suspensions

In experiment 1 a single preparation of tumor agent was irradiated at 2 different dosage levels (0.7×10^5 r and 1.4×10^5 r), and determinations of residual biological activity were made immediately after irradiation (*i.e.* at zero time) as well as 4 hours thereafter in each case. The results are plotted to show the effects of dose at the different times in text-figure 1 (*a* and *b*), and to show the effects of time of standing, after irradiation, at the 2 different dosage levels in text-figure 2 (*a* and *b*). Similar results are shown in text-figures 3–5 for experiments 2 and 3, in



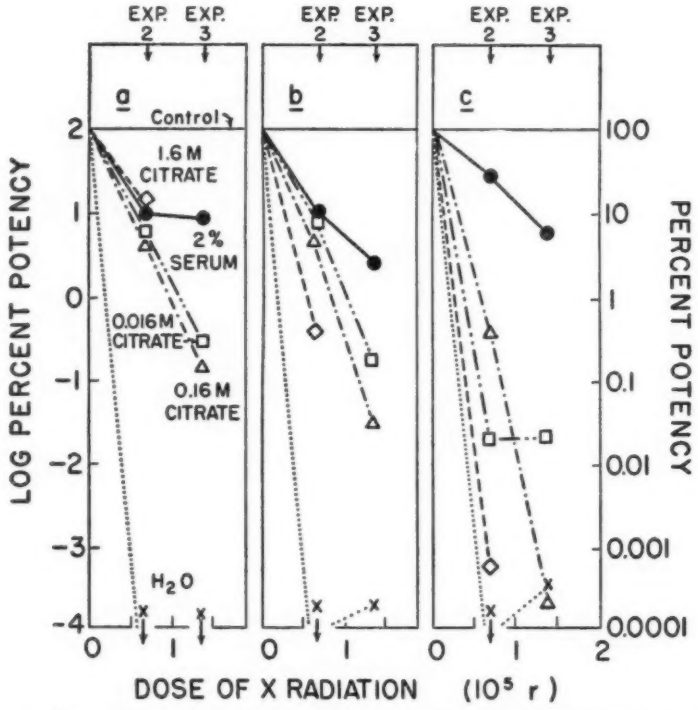
TEXT-FIGURE 2.—Data of experiment 1 and text-figure 1 plotted to show changes with time-following-irradiation.

a. Total dose=0.7×10^5 r.

b. Total dose=1.4×10^5 r.

which separate batches of tumor agent were irradiated with 0.7×10^5 r and 1.4×10^5 r, respectively, and in which observations were carried out at 0, 4, and 24-hour intervals after irradiation.

The plotted points for zero time of these figures demonstrate consistently that citrate in the concentrations employed (*i.e.* 0.016 M to 1.6 M) protected the tumor agent to a considerable extent against immediate inacti-



TEXT-FIGURE 3.—Data of experiments 2 and 3, representing X-radiation doses 0.7×10^5 r and 1.4×10^5 r, respectively. Residual tumor-producing potencies of suspensions of tumor agent in different suspending solutions.

a. Immediately after irradiation.

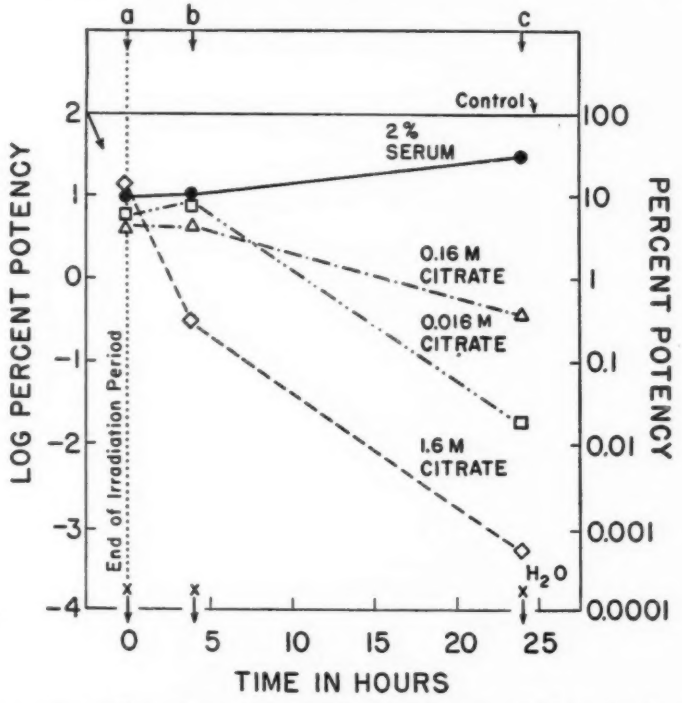
b. After standing for 4 hours at refrigerator temperature (3°–8° C.).

c. After standing for 24 hours at refrigerator temperature (3°–8° C.).

vation by the indirect effects of X radiation, both with total doses of 0.7×10^5 r and 1.4×10^5 r. This is shown by the very marked excess of residual biological activity in the suspensions containing citrate over those which had been prepared with distilled water. The differences between the agent suspensions in citrate solution and in distilled water were highly significant statistically, in spite of the uncertainty associated with the potency values for the latter, weaker, materials (see section on Bioassays). It is evident, therefore, that an abundance of citrate ions protected the agent to a large extent against immediate inactivation by the X radiation. On the other

hand, the 4-hour and 24-hour data of the figures show a progressive decline in residual biological activity with increasing time-following-irradiation, indicating that some slow or delayed reaction capable of inactivating the agent took place in the irradiated suspensions containing citrate. The data for the distilled water preparations are not interpretable in this respect because of their relatively low values and the degree of uncertainty associated with them (see section on Bioassays).

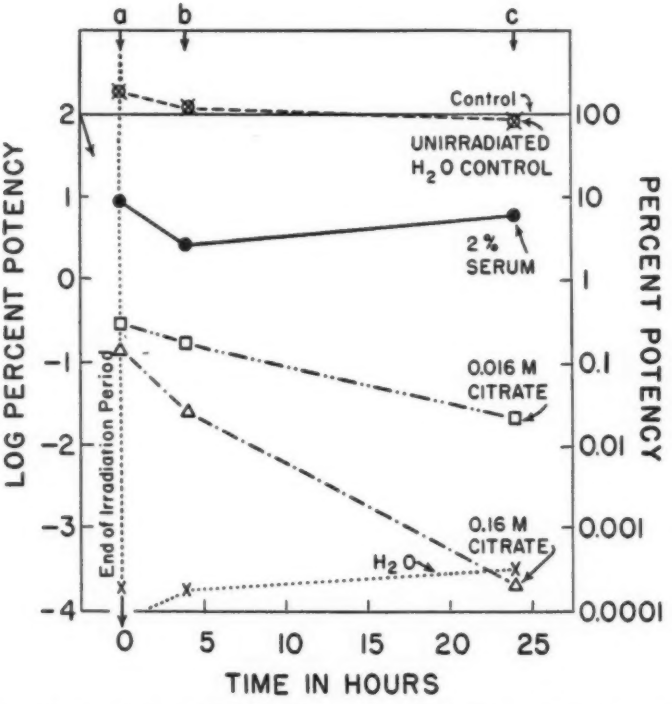
The immediate protection provided by citrate was systematically less than that provided by 2 percent horse serum (under the same dosage con-



TEXT-FIGURE 4.—Data of experiment 2. Residual tumor-producing potencies of suspensions of tumor agent in various suspending solutions, at different times following irradiation. Dose of X radiation $= 0.7 \times 10^5$ r.

ditions) in all tests of experiments 1–3, with the exception of the one involving the 1.6 M concentration in experiment 2. This suggested that the lower concentrations that had been tested, *i.e.* 0.16 M, 0.025 M, and 0.016 M, may have been less than that required to give the maximum protecting effect. Experiment 4 was therefore carried out for the purpose of supplementing the other immediate observations at X-radiation dosage 0.7×10^5 r and extending them to lower concentrations of citrate so that information could be obtained on the nature of the concentration-protection curve. Text-figure 6 shows the results of this experiment (inverted

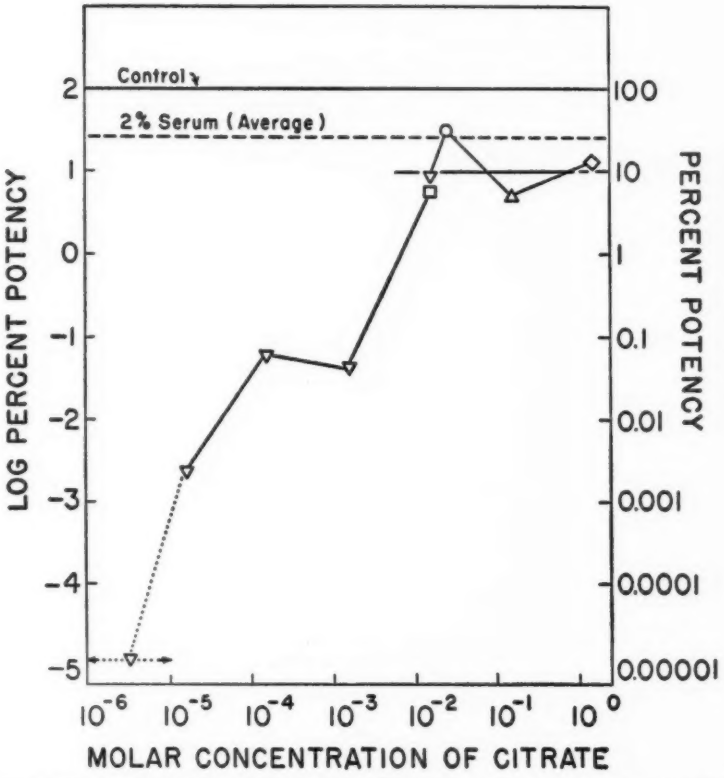
triangles) for citrate concentrations $10^{-5.5}$ M[9] through 1.6×10^{-2} M, together with the results of the preceding experiments (symbols corresponding to those used in text-figs. 1–5) involving the higher concentration range, *i.e.* 1.6×10^{-2} M through 1.6 M. As indicated by the distribution of the plotted points about the short horizontal line drawn through their mean, there does not appear to be any significant correlation between concentration of citrate and the degree of protection in the higher concen-



TEXT-FIGURE 5.—Data of experiment 3. See legend of text-figure 4. Dose of X radiation = 1.4×10^5 r.

tration range, and the curve that would best describe the data as a whole would apparently level off at about 1.6×10^{-2} M. The residual activity dropped rapidly with successive 10-fold decreases in citrate below 1.6×10^{-2} M, however, indicating that there was progressively less protection against indirect irradiation effects at these lower concentrations. The exact nature of the curve relating concentration of citrate and residual biological activity in this region cannot be determined from the data presently available, but there is no indication of a systematic departure from a linear relationship (on the log-log scale of text-fig. 6) down to the lowest concentration investigated.

[9] Estimated concentration of the trace amount present in the "distilled water" suspension as a result of the purification processes involving citrate reagents (see section on Experiments, experiment 4).

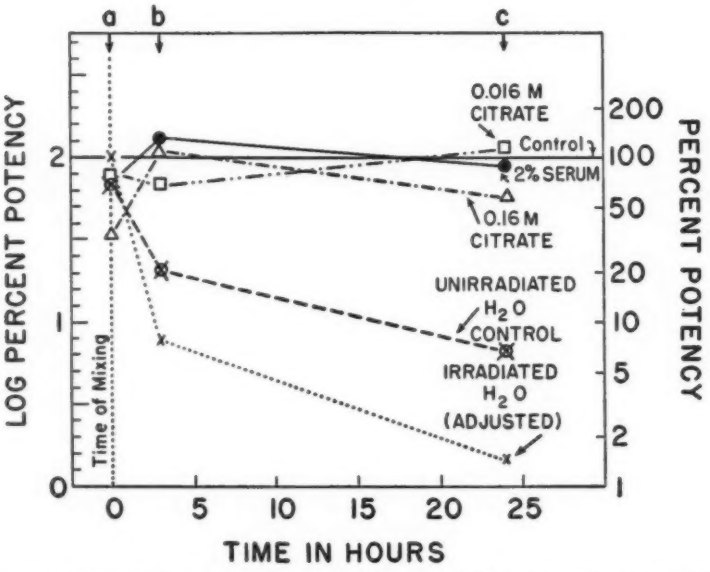


TEXT-FIGURE 6.—Residual tumor-producing potencies immediately after irradiation of suspensions of tumor agent in potassium citrate solutions of different molar concentrations. Dose of X radiation $= 0.7 \times 10^5$ r. ○—data of experiment 1*a*. □, △, and ◇—data of experiment 2*a*. ▽—data of experiment 4. See text and text-footnote 9.

B. Suspensions of Tumor Agent Treated with Previously Irradiated Suspending Solutions

In order to determine whether persistent products capable of inactivating the tumor agent would be formed in citrate solutions during X radiation, the solutions were irradiated with a total dose of 1.4×10^5 r prior to their addition to active tumor agent as described under experiment 5 (see section on Experiments). Text-figure 7 shows the results obtained with 0.016 M and 0.16 M concentrations of citrate as well as with distilled water, and with citrate solution (0.016 M) containing 2 percent normal horse serum.

No significant deviations from their paired controls in unirradiated media (*i.e.* from 2.0 log percent potency) were observed for tumor agent suspensions in citrate or serum-citrate media, either immediately after or during the 24-hour period following addition of the irradiated solutions.



TEXT-FIGURE 7.—Data of experiment 5. Tumor-producing potencies of suspensions of tumor agent in previously irradiated suspending fluids of various types, at different times following their preparation. The ordinate scale is twice that used in the similar text-figures 2, 4, and 5, in relation to the abscissa scale. The dose of X radiation was 1.4×10^5 r in all instances.

These findings indicate that no persistent "toxic" products were present in the previously irradiated citrate or serum-citrate solutions.

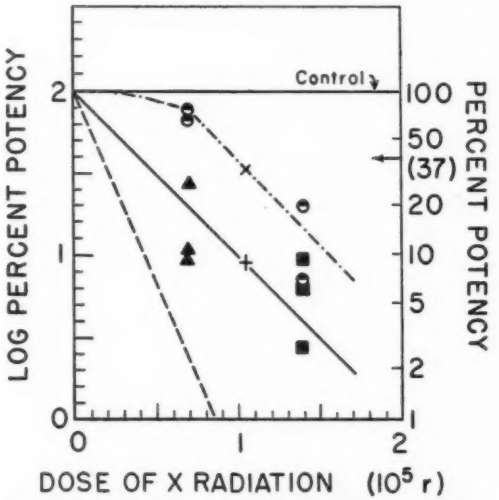
The tumor agent preparation treated with irradiated distilled water showed no significant difference in potency from that of the untreated water control when tested 3 minutes after preparation of the mixtures, thus indicating that no rapidly acting "toxic" substances were present. The observations made after the mixtures had stood for 3 hours, and for 24 hours, were not interpretable with respect to "toxicity" because of the instability of the distilled water control. As shown by the crossed circles of text-figure 7, the water control had probably dropped somewhat in potency at the end of 3 hours, but by 24 hours it was definitely lower, by about a factor of ten.[10] A more rapid decrease in potency in the suspension containing irradiated water than in the control water suspension is indicated by the adjusted values ($\times$ symbols) of text-figure 7 in their relation to the results (crossed circles) for the distilled water control. These findings do not necessarily indicate that slowly acting "toxic" substances were present in the previously irradiated water, because they

[10] As pointed out in the section on Experiments, the distilled water control was found to be unstable in experiment 2 also, although direct assays against a stable standard were not included in this experiment. In another instance in which direct assays were carried out over a 24-hour period, *i.e.* experiment 3, the distilled water control did not decrease significantly (see text-fig. 5). The variation among different tumor agent preparations in distilled water, with regard to their stability, is therefore similar to that previously observed for suspensions in phosphate buffers incubated at 37° for 3 hours (4); no such variations in stability were observed, however, when samples of the same batches of tumor agent were suspended in citrate buffers (4).

could just as readily be interpreted as indicating an enhancement by the irradiated water of the same reactions which caused the decrease in potency of the normal water control.

C. Additional Analyses of Results on Irradiated Tumor Agent Suspensions

Suspensions of tumor agent in solutions containing normal horse serum in addition to citrate.—The results obtained with suspensions of tumor agent irradiated in the presence of 2 percent serum in experiments 1, 2, and 3 represent, primarily, the direct effects of the X radiation [*cf.* Lea (*3*)]. The data may therefore be employed for obtaining information on the size of the unit agent particles (*3*, *1*), or on the state of their physical dispersion in the present preparations as compared with that in various types of preparations previously investigated (*1*). Although only 2 X-radiation doses (0.7×10^5 r and 1.4×10^5 r) were involved in the present studies, a third point on each dose-survival curve is known to be the potency of the control, *i.e.* 2.0 log percent (100 per-

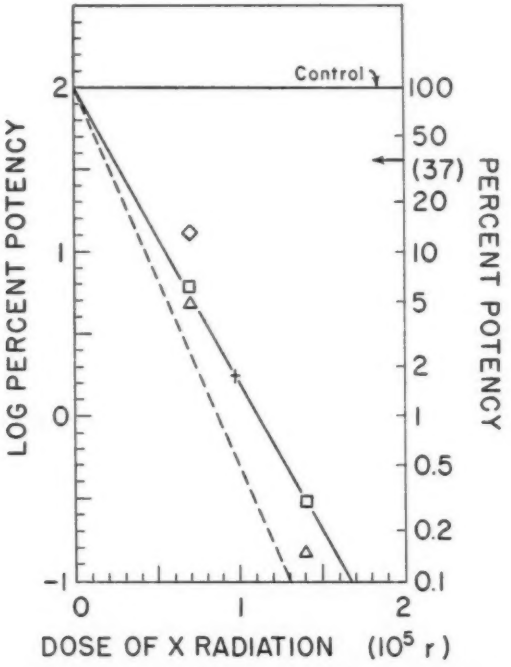


TEXT-FIGURE 8.—Residual tumor-producing potencies of irradiated suspensions of tumor agent in solutions containing 2 percent normal horse serum. ◑—data of experiment 1. ▲—data of experiment 2. ■—data of experiment 3. The x and + symbols represent coordinate means of the respective groups of plotted points indicated by the separate dose-survival curves drawn through them. The broken line at the left represents the expected dose-survival curve for freely dispersed virus particles having a sensitive target diameter of 70 mμ (see text).

cent), at zero dose of X radiation. Since the stability of this agent over a period of at least 24 hours in the presence of serum has long been known and is further confirmed by present data, the additional bioassays of the irradiated suspensions that had been carried out at 4- and 24-hour intervals following irradiation were considered replicate determinations on the respective materials and were included in the analyses. Text-figure 8 shows the plotted points for all pertinent data of experiments 1–3. It is apparent that the results of experiment 1, ◑, deviate systematically not only from the remainder of the plotted points, but also from the straight line which could be drawn through their coordinate means (× symbol) and through 2.0 log percent at zero dose of X radiation. The latter finding indicates that the dose-survival curve (—·—) for the tumor agent preparation of experiment 1 is not exponential, as would be expected for

freely dispersed virus particles [for discussion see references (3) and (1)], but that aggregation, or some other factor, caused distortion of it as in several other types of tumor agent preparations investigated in a previous study (1).

The results obtained with the agent preparations of experiments 2 and 3 (▲ and ■, respectively, of text-fig. 8), on the other hand, show a fairly good fit to the straight (solid) line drawn through their coordinate means (+ symbol) and through 2.0 log percent at zero dose of X radiation. The 37 percent survival dose was found from this computed line to be 4.34×10^4 r, which is practically identical with that (4.37×10^4 r) found in a previous experiment [5A, of reference (1)] in which special measures had been taken to obtain the agent in a highly dispersed state. The estimate of sensitive target diameter, using the same assumptions as before (1), is therefore identical with that previously obtained under similar conditions, namely 49 mμ. This is less than the over-



TEXT-FIGURE 9.—Residual tumor-producing potencies immediately after irradiation of suspensions of tumor agent in citrate solutions. See text, and legend of text-figure 8. The open symbols have the same significance, and represent the same results, as in text-figure 3*a*. The + symbol and the solid and broken oblique lines have the same significance as in text-figure 8.

all diameter of 70 mμ estimated by other indirect methods (7–10), and, as previously pointed out (1), could be interpreted as indicating that not all of the unit particle is radiosensitive [see reference (1) for discussion and further details].

Suspensions of tumor agent in solutions containing citrate only.—Because of the slow inactivating reaction initiated by the irradiation when the "protecting agent" was citrate solution only, and since other, indirect, reactions capable of causing slight immediate reductions in residual potency cannot be ruled out, the X-radiation dose-survival curves obtained with the citrate reagents, in the light of present knowledge, cannot be considered as yielding definitely valid information concerning the size of the

unit biologically active particles. The fact should be mentioned, however, that the X-radiation dose-survival curve determined from the "immediate" data for maximally effective concentrations of citrate in experiments 2 and 3 (text-fig. 9) closely approached that expected for the over-all size of the unit particles as determined by other methods (*7–10*). The data of experiment 1 are not considered in this connection, because of evidence that the agent particles may not have been in a freely dispersed state (see preceding section). The diameter of the sensitive target as determined from the data of experiments 2 and 3 (solid line of text-figure 9) would be 62 mμ, or only slightly less than that (70 mμ) obtained by other indirect methods, and used in computing the "expected" (dash) line of the figure. Whether this is simply a coincidence or whether it represents a "spreading effect" [*cf.* (*3*) pp. 67 and 98] in citrate solutions which is related to total particle size, can be decided only after the true size of the unit active particle has been accurately determined by direct methods, *e.g.* electron microscopy.

Discussion

The most striking result of these investigations was the finding of a slow or delayed inactivating reaction which took place in suspensions of the tumor agent in citrate solutions after they had been irradiated. Although citrate ions appeared to protect the agent to a large extent against immediate inactivation by indirect irradiation effects, the residual biological activity declined progressively over a period of at least 24 hours following the cessation of X radiation. The data show evidence of a dependence of the rate of this slow reaction on the concentration of citrate in the irradiated suspension, but they are inadequate for a quantitative evaluation of the apparent relationship. No such results were obtained when solutions of citrate were irradiated prior to their use as media for suspending the agent, thus indicating that the inactivating reaction was not due to persistent "toxic" products formed by irradiation of the citrate media *per se*. Nor was the reaction observed when the irradiated suspensions contained 2 percent normal horse serum in additon to citrate. These findings, together with the fact that the maximum protecting action of citrate, at any concentration, was systematically less than that provided by 2 percent serum, suggest that the tumor agent may have been damaged to some extent immediately, when irradiated in the presence of citrate reagent only, and that one of the manifestations of this damage was a decrease in the stability of its tumor-producing property [*cf.* (*11*)]. The possibility should not be overlooked, however, that the apparent discrepancy between serum and citrate, in the amount of immediate protection they provided in their maximally effective concentrations, could be due to the fact that the size of the sensitive target was actually, or effectively, greater in the citrate reagents than in the solutions containing serum.

Another finding of interest was the variation among different batches of tumor agent in their responses to irradiation. Although electron micrographic studies were not made on the materials used in the present experiments, preliminary studies on other materials prepared in a similar way (see section on Materials and Methods) had indicated that they were relatively free of aggregated material. The fact that the dose-survival curve for direct irradiation effects in experiment 1 was similar to that obtained with aggregated suspensions in a previous study (*1*), suggests

that either the preparative procedure described herein does not always yield aggregate-free material, or that some other type of factor caused distortion of the curve in experiment 1. Among other possible factors previously discussed (*1*) was dissociation of the agent particles from some inhibiting factor with which they had been combined and which had masked their biological activity to some extent. If the latter type of factor were involved it should be possible to enhance the biological activity of such masked preparations by dissociation procedures that would not destroy any of the agent. An obvious problem, prerequisite to the further use of this agent as a quantitative test object for investigating the biological effects of irradiation at the "macromolecular" level, is that of further purification and standardization of the agent materials prepared from different source lots of tumor tissue.

Summary and Conclusions

Suspensions of the agent of chicken tumor I (Rous sarcoma virus) in citrate solutions of varying molar concentration were irradiated with total doses of 0.7×10^5 r or 1.4×10^5 r of X radiation, and determinations of residual biological activity were made at 0, 4, and 24-hour intervals following irradiation. Similar determinations were made, also, on irradiated suspensions of the agent in distilled water and in citrate solution containing 2 percent normal horse serum. Additional control observations involved the treatment of active preparations of the tumor agent with previously irradiated suspending solutions, the same as above.

It was found that citrate in 1.6×10^{-2} M or higher concentration in the suspending solution protected the agent to a large extent against immediate inactivation by indirect effects of the X radiation, but that a slow or delayed reaction followed which resulted in a progressive fall in residual biological activity over a period of at least 24 hours. The immediate protection by citrate, in maximally effective concentrations, was systematically less than that provided by normal horse serum. Successive decreases in citrate concentration below 1.6×10^{-2} M gave progressively less immediate protection against the indirect irradiation effects, down to the lowest concentration tested, namely $10^{-5.5}$ M.

The findings are discussed and certain of the data involving direct irradiation effects are compared with similar data previously obtained.

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An Analysis of Dose-Response for Animals Treated with Aminopterin and Citrovorum Factor [1,2]

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In previous studies with mice, a linear relationship was observed between the median lethal dose (LD_{50}) of aminopterin and the dose of concomitantly administered citrovorum factor (CF)[5] (*1*). This observation suggests that on concomitant administration of CF and aminopterin, the dose-mortality relationship may be described by the equation for a modified probit plane:

$Y = a + b \log [A/(C + CF)]$, where
Y = probit of percent mortality
A = moles aminopterin/kg. administered
CF = moles CF/kg. administered
C = endogenous reserve of protection, expressed as moles CF/kg. administered simultaneously with aminopterin
b = probit slope constant; probits per 10-fold increase in $A/(C + CF)$
a = probit of percent mortality when $A = C + CF$

Conformance with the modified probit plane model would be indicative of competitive antagonism. Under the model, for any selected percent mortality response (p), a linear relationship would obtain between the dose of aminopterin and the dose of concomitantly administered CF. Specifically,

$$A = K_p (C + CF)$$

where K_p = antilog $[(Y_p - a)/b]$, and Y_p is the probit corresponding to

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[2] The authors wish to acknowledge the interest and helpful suggestions of Dr. Jacob Colsky during the course of these investigations. They also wish to acknowledge the technical assistance of Don Dennis, Vera Rhein, and Ina R. Hughes.

[3] *Present association:* Laboratory of Chemical Pharmacology, National Cancer Institute.

[4] National Institutes of Health, Public Health Service.

[5] Leucovorin, designated in this paper as CF. Leucovorin (CF) and aminopterin were kindly provided by the Lederle Laboratories Division of the American Cyanamid Company. The Leucovorin (CF) contained 20 million units per ml., equivalent to 3 mg. CF per ml.

mortality percentage p. K_p is the slope of the aminopterin-CF line for percentage mortality p.

The current investigation was undertaken in order to provide a rigid test of the validity of the foregoing dose-mortality relationship in mice, over a wide range of administered CF.

Methods

An experiment was conducted, described earlier (*1*), employing CF and aminopterin administered concomitantly. The mice were assigned at random to the different treatment groups, but the treatments were not performed randomly. The data of this experiment are here supplemented by the results of additional work in which the maximum CF dosage previously reported (*1*) was increased fourfold.

In addition to the previously described experiment, a wholly randomized experiment was conducted [6] with 320 strain A (NCI) male mice weighing 17 to 23 grams. The general methods were the same as those previously described (*1*). However, not only were the animals chosen at random, but also the dosages were administered at random. The randomizing procedure was as follows: Prior to treatment, 320 file cards were prepared, one for each animal required in the experiment. On each file card were entered the dosage to be administered and the experimental group number. The cards were randomized and then numbered serially to correspond to the order of treatment. Mouse numbers were assigned to the members of each experimental group in the order in which the cards appeared in the pack. The mice were picked at random from a common pool, weighed, and the designated dosage administered in accordance with the established serial order of the cards. The mice were then earmarked with the proper mouse number and placed in the appropriate group cage. All treatments were accomplished within a three-hour interval in a single day. The CF dosage range previously reported (*1*) was increased fourfold. The animals were observed daily for mortality for a period of 11 days following the day of treatment. The pooled experiments, extended over the wider dosage range, are designated below as Series I; the completely randomized experiment is designated Series II.

Results

The data of Series II, and calculated results for both Series I and II, are summarized in tables 1 and 2, and in text-figures 1 and 2. The results obtained for the single randomized experiment (Series II) did not differ substantially from those obtained with the earlier mixture of experiments, which for the present study had been expanded to cover a wider CF range (Series I). The linear increase in the LD_{50} of aminopterin continued to be manifest at the higher doses of CF. There resulted a more than twelvefold increase in the LD_{50} of aminopterin when adminis-

[6] It was considered desirable to conduct this additional wholly randomized experiment, since the earlier results were based on a mixture of experiments, done at different times. In addition, although no significant differences were noted in their dose-response relationships, the results with two strains of mice had been pooled in the earlier experiments (*1*).

tered with the highest dose of CF employed, as compared with the LD_{50} for aminopterin without added CF (table 1, text-fig. 1).

TABLE 1.—*Results of randomized experiment (Series II)**

Aminopterin dosage (10^{-6} moles A/kg.)	Citrovorum factor dosage (10^{-6} moles CF/kg.)					
	0	3.96	7.93	15.9	31.7	63.4
	Number of deaths within 11 days among 10 treated mice					
4.04	1					
6.06	0					
9.08	8	0				
13.6	6	1	1			
20.4	5	1	1	0		
30.6	10	2	3	1		
46.0		10	8	4	1	
69.0			8	8	3	1
103				10	7	5
155				10	10	5
233					10	8
349						9
Calculated LD_{50} of aminopterin (10^{-6} moles A/kg.):						
From common probit slope lines	11.2	33.2	36.3	49.5	78.8	138
From individual probit slope lines	11.2	31.6	36.6	49.7	79.7	135
Calculated probit slope—probits per 10-fold increase in A:						
Common probit slope			4.22			
Individual probit slope	2.91	5.58	3.74	6.80	6.33	3.24

*320 mice assigned and treated at random on a single day. No deaths occurred in 11 days among 9 untreated mice.

TABLE 2.—*Maximum likelihood estimates of parameters relating probit of response linearly to the logarithm of the ratio of administered aminopterin to administered plus endogenous equivalent of citrovorum factor (modified probit plane)*

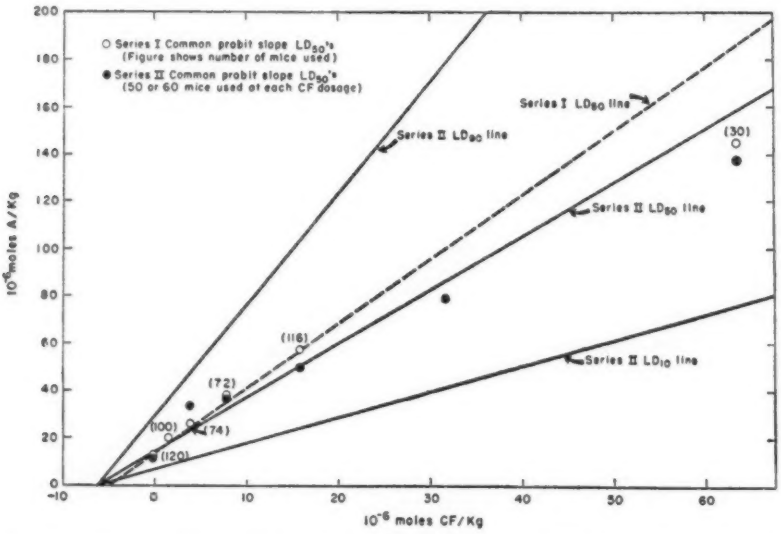
	Series I	Series II
LD_{50} inhibition index* $K_{50}=A_{50}/(C+CF)$. Ratio of LD_{50} of aminopterin in moles/kg. to added plus endogenous equivalent of CF in moles/kg.	2.72±0.25†	2.28±0.27†
Endogenous reserve equivalent $=C$. 10^{-6} moles CF/kg.	4.89±0.75	6.03±1.29
Probit slope $=b$. Probits per 10-fold increase in $A/(C+CF)$.	4.13±0.23	4.05±0.55

*Equivalent to increase in LD_{50} of aminopterin in moles/kg. per mole/kg. increase in citrovorum factor.

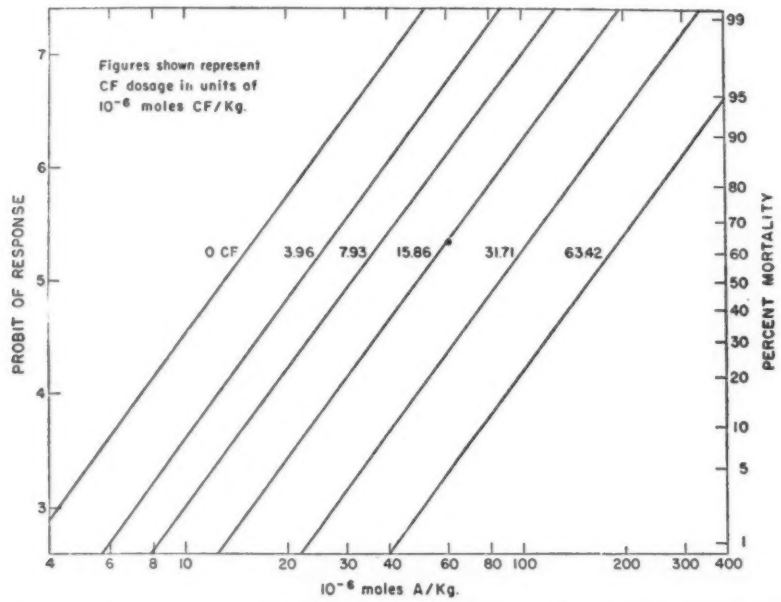
†Standard error of estimate. For Series II standard errors have been stepped up by a factor of $\sqrt{2.06}$ to reflect heterogeneity of results.

Table 1 shows, for Series II, the number of mice that died of 10 treated mice at each CF-aminopterin dosage combination. Probit curves of three types were fitted to these results.

(a) *Individual dosage-response curves to aminopterin for each level of CF.*—Each CF level is assumed to have its own probit slope and LD_{50}. These were fitted using one cycle of the maximum likelihood iterative procedure (*2*), and the results are shown at the bottom of table 1.



TEXT-FIGURE 1.—Linear relationship between LD_{50} of aminopterin and added CF from the fitted modified probit plane for Series I and II; LD_{10} and LD_{90} lines for Series II.



TEXT-FIGURE 2.—Response curves to aminopterin with varying CF, from the fitted modified probit plane, Series II.

(b) *Common probit slope dosage-response curves to aminopterin for the various levels of CF.*—The various CF levels are assumed to have individual LD_{50}'s but a common probit slope. These were fitted using one cycle of the maximum likelihood common slope iterative procedure (*2*). The results are shown at the bottom of table 1, and in addition the individual LD_{50}'s are plotted in text-figure 1.

(c) *Modified probit plane response curve to aminopterin plus CF.*—The various CF levels are assumed to have a common probit slope with the LD_{50}'s (or any other designated percentage lethality) of aminopterin linear against the CF dosage. Two cycles of the maximum likelihood iterative procedures developed[7] for this type of response curve were used to fit the data. The values defining the fitted curve are shown in table 2, and text-figure 1 illustrates the fitted linear relationship between the LD_{50}, LD_{10}, and LD_{90} of aminopterin with CF. Text-figure 2 shows the probit response curves for the different CF levels used corresponding to the fitted modified probit plane.

For Series I a similar set of probit curves was fitted, but the results are not shown in detail here. The LD_{50}'s from the common probit slope dosage-response curves are plotted in text-figure 1. Text-figure 1 also shows the fitted linear relationship for this series between the LD_{50} of aminopterin and CF, and the values defining the fitted modified probit plane are given in table 2.

A statistical analysis is presented in detail in the appendix and summarized in table 3. The analysis indicates that the data of the wholly randomized experiment (Series II) conform to the modified probit plane model. Analysis of the data of the pooled experiments (Series I) gives essentially the same result. However, with Series I there is a borderline indication of lack of conformity to the model. This may be due to the fact that Series I is a combination of experiments done on different days and under different conditions.

When death occurred following treatment with aminopterin, with or without CF, the day of death did not appear to be closely correlated with the dosage of aminopterin employed. Most deaths occurred by the sixth day, with none occurring before the third day following treatment.

Discussion

The toxicity of aminopterin is a function of the dosage of inhibitor and substrate employed, and the dose-response relationship is adequately described by the equation for a modified probit plane. For any selected percentage mortality a linear relationship obtains between the dose of aminopterin and the dose of CF employed. Conformance with the model supports the earlier suggestion (*1*, *3–5*) that a competitive relationship exists between aminopterin and CF in enzymatic transformations involving CF.

[7] To carry out the analysis of the data it was necessary to develop maximum likelihood iterative procedures for fitting the modified probit plane. These procedures will be reported separately by one of the authors (N. M.). In the previous report (*1*) LD_{50}'s were computed by the method of Litchfield and Wilcoxon (J. Pharmacol. & Exper. Therap. 96: 99–113, 1949), and in turn a weighted least squares straight line was fitted to the computed LD_{50}'s.

The current study indicates that, in the mouse, the analysis of dose-response relationships may provide a basis for "inhibition analysis" (*6–8*). The inhibition index (*7*) may be expressed as $A_{50}/(C+CF)$, the ratio of inhibitor LD_{50} (A_{50}) to the substrate added (CF) plus a factor (C) representing the endogenous protection expressed as CF. The inhibition index, expressed in this form, is constant, and is the slope, K_{50}, of the line expressing the molar ratio of aminopterin LD_{50} and CF dose (table 2). A similar relationship would obtain for any selected mortality response.

Some evidence has been provided that, as with unicellular organisms (*7*), exogenous factors other than the corresponding metabolite may alter the inhibitory effect of the antimetabolite for the animal and may affect the inhibition index. *a*) Folic acid (FA) when administered early, apparently acting as a precursor of CF, gave the appearance of protecting against aminopterin toxicity in a competitive manner (*1*). The LD_{50} of aminopterin with concomitantly administered CF was approximately doubled when, in addition, FA was administered one hour before the aminopterin (*1*). This would result in an apparent twofold increase in the inhibition index. *b*) Prior administration of aminopterin, apparently by causing irreversible enzymatic alteration, resulted in diminished protection by FA or CF (*1*). Such irreversible alteration resulting in decreased protection by metabolite, would also result in a decrease in the inhibition index.

It has been reported that administration of FA or CF may interfere with the tumor-inhibitory effects of aminopterin or 4-amino-N^{10}-methylpteroylglutamic acid(a-methopterin) (*9–13*). These observations have been confined to the effect of FA and CF on the therapeutic action of a dose of analogue in the range of the maximum therapeutic dose on multiple treatment (*9–13*). The current investigation may provide a basis for determining to what extent tumor growth may be influenced by the toxicologic interrelationship exhibited on combination treatment with aminopterin and CF. It is suggested that "inhibition analysis," based on dose-response relationships in the mammalian organism, may provide a tool to help clarify the role of antagonists in influencing tumor growth.

Summary

The dose-mortality relationship for aminopterin and concomitantly administered citrovorum factor (CF) was investigated in mice over a wide range of dosage of the drugs. The relationship appears to be adequately described by an equation for a modified probit plane model. The probit of response to combination treatment with aminopterin plus CF is linear with the logarithm of the ratio of aminopterin to the administered CF plus a constant. The constant is the CF equivalent of the endogenous protection present in the mouse.

This relationship is fitted to the experimental data and estimates of the parameters defining the modified probit plane are made. The validity of the modified probit plane model is tested statistically and is found to be consistent with the data.

The results are discussed with reference to "inhibition analysis" in the mammalian organism.

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Appendix

Statistical Validity Test

The results of the statistical validity test are summarized in table 3 (see next page).

The χ^2 goodness-of-fit test is used to provide a statistical significance test of the validity of the biological model of a modified probit plane response curve. χ^2 is given by

$$\chi^2=\sum(r-n\hat{p})^2/np(1-p)$$

where r among n animals receiving a specific treatment are observed to respond, and $\hat{p}$ is the calculated proportion expected to respond under the model. Ordinarily p and $\hat{p}$ are the same but in the present case it was necessary to distinguish between them as indicated below.

TABLE 3.—*Analysis of* χ^2

Series I	χ^2	Degrees of freedom	Mean Square	F	P
Source of variation:					
All departures from the modified probit plane	*45.50*	*41*	1.11	1.11*	n.s.‡
Departures of individual probit lines from the modified plane.	*16.73*	*9*	1.86	2.07†	n.s.
Departures from linearity of LD_{50}'s assuming equality of slopes	4.99	4	1.25	1.39†	n.s.
Departures from equality of probit slopes	11.74	5	2.35	2.61†	$P<.05$
Departures from individual probit lines—residual error	*28.77*	*32*	0.90	0.90*	n.s.
Series II					
Source of variation:					
All departures from the modified probit plane	*59.71*	*29*	2.06	2.06*	$P<.001$
Departures of individual probit lines from the modified plane.	*25.99*	*9*	2.89	1.71†	n.s.
Departures from linearity of LD_{50}'s assuming equality of slopes	14.89	4	3.72	2.20†	n.s.
Departures from equality of probit slopes	11.10	5	2.22	1.31†	n.s.
Departures from individual probit lines—residual error	*33.72*	*20*	1.69	1.69*	$P<.05$

* F calculated relative to unity.
† F calculated relative to residual mean square.
‡ n. s.: not significant, $P>.05$.

For each combination treatment of aminopterin and CF three values of $\hat{p}$ were calculated: $\hat{p}_1$, corresponding to the fitting of individual probit curves for each CF level; $\hat{p}_2$ corresponding to the fitting of common probit slope response curves; and $\hat{p}_3$ corresponding to the modified probit plane response curve. For each set of $\hat{p}$'s a χ^2 was calculated, $\hat{p}_3$ being used for p in the denominators in all χ^2's.

While χ^2_3 (corresponding to the modified probit plane) does provide a valid measure of the goodness of fit of the modified probit plane, a more specific test of validity of the modified probit plane model is provided by $\chi^2_3 - \chi^2_1$ (χ^2_1 corresponding to the individual probit curves). If $\chi^2_3 - \chi^2_1$ were significant it would indicate that a significantly better fit is provided by the individual probit curves, and would suggest that the modified probit plane model is invalid. The quantity $\chi^2_3 - \chi^2_1$ can be subdivided into two parts, $\chi^2_3 - \chi^2_2$ and $\chi^2_2 - \chi^2_1$. These two parts provide a more detailed test of the validity of our model.

Under the modified probit plane model the LD_{50} of aminopterin (and all other LD's) is linear with CF, and the probit slope is constant from CF level to CF level. $\chi^2_3 - \chi^2_2$ tests the linearity of the LD_{50}'s assuming the probit slopes constant, while $\chi^2_2 - \chi^2_1$ tests the constancy of probit slopes whether or not the LD_{50}'s are linear. χ^2_1, by itself, provides a statistical test of the validity of the simplest probit model, namely, a separate probit slope and LD_{50} of aminopterin for each CF level. However, in dose-response work, the simplest probit model is customarily accepted as valid, and χ^2_1, instead of being used to test the validity of the model, may be used to provide a measure of experimental heterogeneity. As such, it may be used as an error term in testing the remaining χ^2's.

For Series I, χ^2_3 was 45.50 with 41 degrees of freedom (table 3), indicating satisfactory

fit, while χ^2_1 was 28.77 with 32 degrees of freedom, indicating experimental homogeneity. The partition of χ^2 indicated no significant departure from linearity of the LD_{50}'s but did indicate significant ($P<.05$) departure from constancy of the probit slopes. The individual CF probit slopes did not, however, show any definite pattern with changing CF. This finding of borderline significance should perhaps be discounted because of the double risk involved in testing linearity of LD_{50}'s and equality of probit slopes separately. Moreover, since in Series I experiments done on different days were combined, we should expect to find, if there are day effects, steeper probit slopes for CF levels used in only one experiment than for CF levels used in several experiments. This would occur if the modified probit plane held only on a single day but shifted from day to day.

If important day effects did exist and did account for the probit slope differences observed for Series I it would render the estimates made from Series I invalid. It was to obtain more valid estimates of the probit plane parameters and to put the investigation of the probit plane relationship on firmer ground that Series II was performed. Contrary to expectation Series II gave substantially the same estimates as Series I, especially on the probit slopes, even though Series II was performed on a single day. χ^2_3, however, was quite large (59.71 with 29 degrees of freedom, $P<.001$); but so was χ^2_1 (33.72 with 20 degrees of freedom). Relative to the lack of homogeneity indicated by χ^2_1, there was no significant indication of non-linearity of LD_{50}'s or inconstancy of probit slopes.

The lack of homogeneity found in Series II arose principally from the results for 0 CF and 3.96×10^{-6} moles CF/kg., at which CF levels there were, respectively, inversions in percentage response, and a sudden change in percentage response (table 1). While in both series the LD_{50} at the highest CF level was somewhat below that indicated by the fitted modified probit plane, the difference was not important when compared with the large standard error of the LD_{50} that obtained at this CF level. In table 2 the standard errors for the estimates made from Series II have been stepped up by a factor $\sqrt{59.71/29}=\sqrt{2.06}$ to reflect the lack of homogeneity found in this experiment.

It should be noted that χ^2 was calculated without combining different dose levels even when expected frequencies were quite small. This procedure does not change the expected value of χ^2 but will affect its distribution. It was felt safer not to combine frequencies and have the distribution of χ^2 change, than to use combinations of frequencies which might obscure the very effects χ^2 was intended to bring out.

Electron Microscopic Study of Epidermal Carcinoma Induced by Methylcholanthrene in the Mouse[1]

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The object of this investigation was to evaluate electron microscopy of thin tissue sections as a new tool in pathologic histology. The technique of electron microscopy has recently been developed to such an extent that examination of histologic sections with this instrument has become a relatively simple procedure. The danger of major artefacts has been reduced to a minimum. Changes in tissue structure resulting from embedding in methacrylate or other plastics are inevitable but not more extensive than those occurring after the usual embedding in paraffin. Examination under the electron microscope does not damage the object if exposure is limited to short periods of time. In other words, the technical handicaps in regard to denaturation of the material in electron microscopy are by no means different from those of microscopy with the usual embedding methods. On the other hand, electron microscopy offers great advantages for certain detailed investigations in view of the much greater resolving power and the elimination of all staining procedures, which in themselves always produce artefacts.

We have decided to limit our present investigation to one object, *viz.*, to the cytoplasmic structures of the cells of the epidermis. It seemed promising to select for this purpose the changes in mouse skin resulting from repeated application of methylcholanthrene. As is well known, mouse skin reacts to repeated methylcholanthrene applications with very pronounced hyperplasia, eventually resulting in true neoplasm. This experiment, furthermore, offers a possibility to observe morphologically the transition and metamorphosis from hyperplastic epithelium to the epithelium of a genuine tumor.

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Materials and Methods

The animals used in this experiment were: *a*) at Washington University—for the hyperplasia experiment, young white (Swiss) mice, and for the transplantation experiments with methylcholanthrene epithelioma, gray mice (strain C57L); *b*) at the National Institutes of Health in the United States, young mice of strain A.

Methylcholanthrene was used in 0.6 or 0.25 percent solution in benzene, applied once a week to the skin of the back of the animal. The histologic material was simultaneously embedded in paraffin (for ordinary light microscopy) and in plastic for electron microscopy (EM). Paraffin sections were fixed and stained by usual methods. EM sections were fixed in buffered osmic acid and embedded in methacrylate (plastic). Thin sections of about 0.1 micron were cut and shadow-cast with nickel. All EM photographs were made with a 50 kv. RCA electron microscope.

Results

By the above method, a diffuse hyperplasia of the epidermis of the skin can be produced after a few paintings. In many experiments, the skin showed microscopically slight thickening even after small doses. Histologically, this corresponds to a thickening of the epidermis up to ten rows of cells; these cells show marked signs of proliferation. Prolonged application of methylcholanthrene results first in the formation of small papillomas and later of large skin tumors, which often ulcerate. Microscopically, it is impossible to determine whether carcinoma has developed while no detectable metastases occur.

We have selected from our material a certain group of cases useful for a study of electron microscopy and suitable for demonstrating the different stages of proliferation of the epithelium. The *first stage* may be termed *simple hyperplasia*, a diffuse thickening of the epidermis without transgression of the tissue borders and without atypical cell forms.

Normal mouse skin has a very thin epidermis with generally only two layers of cells (fig. 1), a basal layer of cuboidal cells and a granulated top layer showing partial cornification. With simple hyperplasia there is a thickening up to ten rows of cells; large nuclei with large nucleoli are signs of proliferation. Quite often one can find cells with two nuclei; the mitoses are also more numerous. The cytoplasmic structures—*viz.*, intercellular bridges and tonofibrils—are particularly distinct in simple hyperplasia. For example, mouse #805 was given four applications of 0.25 percent methylcholanthrene. The skin showed a slight degree of hyperplasia, an increased tonofibril system, and very marked intercellular bridges. Figure 2 shows how the tonofibrils run across the cytoplasm in all directions. In the center of the cell they are often united in large bundles, whereas at the surface they are more dispersed. Where they enter an adjacent cell one can often observe, within the intercellular bridges, cufflike thickenings that probably represent desmosomes, as seen in normal tissue. In figure 3 the tonofibrils appear much finer, and their passage into the adjacent cells is especially clear. The intercellular

bridges are not visible in this case but the cell borders are more pronounced due to the presence of the desmosomes, which are arranged in rows. Even more pronounced are the cell borders in figure 4, where the thickening of the fibers is very marked. It is very probable that the differences represented in these three photomicrographs are related to different positions of the cells within the thickened epidermis. Since the latter two, figures 3 and 4 (especially 4) show large amounts of keratohyalin, they probably represent cells from a more external zone than those of figure 2. Intercellular bridges—*viz.*, bridges that cross a fissurelike interspace—are only visible in figure 2. The nuclei show no differences in these three pictures. The essential fact represented is the thickness and the relative regularity in the arrangement and distribution of the cytoplasmic fibrils. Schaffer (*1*) surmises that the fibril systems of all cells are connected with each other, forming a functional unity, the arrangement of which is related to the external mechanical influences. We agree with his opinion that these are manifestations of a basic, probably functional, differentiation of the cytoplasm and that they are physiological products of differentiation of the epidermis. In normal human skin, these fibers increase in number with progressive physiological differentiation—*viz.*, toward the surface of the skin—and again the intercellular bridges disappear in the top layers, according to a decrease in intercellular fluid. One may suspect that the intercellular bridges are formed by the cells being separated from each other by the intercellular fluid. During this process the differentiated cell connections—*viz.*, the tonofibrils—remain intact since they are fortified by the desmosomes. This also explains why the epidermal cells in the top layers appear to have numerous tonofibrils with desmosomes, but no intercellular bridges. There is no doubt in my mind that intercellular bridges are a cytoplasmic structure, and one may assume that they are formed either of tonofibrils or amorphous cytoplasm or of both, the tonofibrils being perhaps surrounded by a coating of undifferentiated cytoplasm.

The *second stage* is represented by excessive hyperplasia with transition into true tumor. For example, mouse #408 was given 15 applications of 0.25 percent methylcholanthrene. The skin of the back showed macroscopically a tumor one-half centimeter in diameter, ulcerated on top. There were no metastases. Histologic examination by ordinary microscope showed excessive proliferation of the epidermis in the form of plump, solid strands (fig. 5). These strands generally show large basal layers of polymorphous, poorly differentiated cells with relatively large nuclei and little cytoplasm. Mitoses are moderate in number. Toward the center one may see epidermal differentiation with a very marked formation of tonofibrils, with layers of granulated cells and excessive cornification.

In a few areas of the sections there is a loosening of the cells in the basal layers and a segregation or "dropping" of atypical fusiform cells into the underlying connective tissue ("Abtropfung"). As shown in figure 6 these fusiform cells, which separate, have elongated nuclei with a great deal of chromatin and large, often multiple, nucleoli. Their cytoplasm is greatly reduced. In the neighborhood of these segregation centers there

are independent nests of cells within the connective tissue showing polymorphy, loss of structure, and all other signs of malignancy. To summarize, this case with ordinary light microscopy shows extensive hyperplasia of the epidermis with transition into true carcinoma; *i.e., hyperplasiogenic carcinoma.*

On examination of this tissue with the electron microscope, many sections showed areas in which the cells contained well-developed cytoplasmic fibrillar structures, corresponding to those found in simple hyperplasia. In addition, however, other areas were found in which the intercellular spaces were enlarged. They formed a complete ring of large vacuoles around the thin cells, which had almost no cytoplasm. Within the cytoplasm, a few plump fibers appeared to be present (fig. 7), but this cytoplasm certainly demonstrated a high degree of loss of mass and structure. The cells consisted of an enlarged nucleus with large nucleolus, little cytoplasm, and a thorny crown of intercellular bridges which in some places were very fine, in others very thick. Figure 7 shows those intercellular bridges mostly in the form of elongated filaments of undifferentiated cytoplasm, and in general gives the impression of a primitive epithelium. The loss of structure is even more apparent in figures 8 and 9, where the large vacuoles between the intercellular bridges are the most prominent feature. To summarize, electron microscopic examination in this case showed, in accordance with regular light microscopy, the picture of hyperplasia as well as that of anaplasia (dedifferentiation) with loss of cytoplasmic structure.

The *third stage* of proliferation is represented (figs. 10–12) by sections of a transplanted methylcholanthrene-induced carcinoma of the ninth passage in gray mice of strain C57L. This is a rather slowly growing carcinoma that does not produce metastases. Histologically, it is a parakeratotic, moderately dedifferentiated carcinoma (fig. 10) with a rather quiescent cell pattern, but with clear polymorphism and increased number of mitotic figures. Very little stroma is present. In the enlarged basal zones of the cellular network, no typical basal cells could be found, and the nuclei lie quite tightly together. The electron microscope reveals a very uniform picture, as in figures 11 and 12. Figure 12 shows the great density of nuclei. These nuclei, generally well preserved and often containing several nucleoli, are separated from each other only by very sparse cytoplasm. Normal structures of the type of tonofibrils are absent, and intercellular bridges are hardly visible. In figure 12, however, the pericellular systems of vacuoles are recognizable, but there is a lack of any higher degree of differentiation.

The *fourth and last stage* is represented by an extremely dedifferentiated methylcholanthrene-induced carcinoma of the skin of the back (mouse #626, of strain C57BL, which received 15 applications of methylcholanthrene—chosen from a series of experiments of Dr. Andervont). This is a very malignant metastasizing tumor, whose histologic nature is open to question. The lung metastases showed the picture of a spindle-cell sarcoma. On the other hand, the primary tumor of the skin certainly showed,

as one component, the epithelial structure of a cornifying squamous-cell carcinoma (see fig. 13). The formations resembling "keratin pearls," numerous in the center of the tumor, were gradually replaced toward the periphery by a purely cellular mass of extremely dedifferentiated spindle cells that generally consisted of long polymorphous nuclei and very little cytoplasm. An intercellular substance could not be demonstrated. Therefore, we are confronted with the differential diagnosis of carcinosarcoma *versus* anaplastic spindle-cell carcinoma. In view of the fluctuant and gradual transition from one component to the other, I consider this tumor to be a highly anaplastic dedifferentiated spindle-cell carcinoma. The electron microscope photographs of this tumor are very instructive. They show advanced repression of structure. The nuclei in all three pictures (figs. 14–16) are well preserved and have sharp borders with large and more numerous nucleoli, but the cytoplasm has lost most of its typical structure. Also, the volume of cytoplasmic material is diminished. The very sparse cytoplasm is partly arranged in the form of filaments or fine granular materials, but most of it has no definite relation at all to the nucleus. Since the preparation was fixed with osmic acid, it is possible that the granular material represents a protein-containing product of fixation, similar for example to the electron microscopic picture of coagulated gelatin. In any case, the electron microscope shows evidence of lack of typical cytoplasmic structure. The fact is even more prominent when these photomicrographs are compared with those of hyperplastic skin.

Discussion

The question asked at the start of this investigation—whether the electron microscope is suitable for thin sections in histopathology—therefore can be answered positively. We are convinced that this method enlarges further the range of morphologic pathology, especially when certain "childhood diseases" of the new method are eliminated. However, it is wrong to believe that this method is suitable only for virus research, a field explored intensively at the present time. There are many other histologic and cytologic problems that may be solved by the high resolving power of the electron microscope.

The methylcholanthrene-induced carcinoma of mouse skin is certainly a suitable object for histologic and cytogenetic tumor studies. The experimental attempt to solve certain problems of cytoplasmic differentiation by the use of the electron microscope appears to be successful in view of the results of this investigation. However, only a large-scale study of this type will make it possible to obtain a continuous picture of tumor evolution from which conclusions regarding the pathogenesis of this tumor may be drawn. Nevertheless, I believe that the present findings are worth while, since they are based on a great number of individual examinations. Lester S. King (*2*) has attempted to investigate the effect of podophyllin on mouse skin, especially upon the intercellular bridges and fibrils, and has compared his findings with those obtained with methylcholanthrene. He found, even in moderate hyperplasia produced by

methylcholanthrene, a pronounced appearance of intercellular bridges ("the bridges become prominent"), and this feature was most apparent in true methylcholanthrene carcinoma. King has shown that the methylcholanthrene carcinoma shows great variation in differentiation. To demonstrate tonofibrils, highly differentiated tumors were most suitable. Tumors with slight differentiation showed only very poorly developed fibrils. This seems to be in accordance with the findings presented herein. However, I cannot agree with King when he states that the malignant cells show a very pronounced development of tonofibrils. In my opinion this statement is contradictory, since King himself has demonstrated that the presence of fibrils seems to be in directly proportionate relationship to the differentiation of the tumor, which means that the fibrils disappear with progressive dedifferentiation, or in other words, with increasing malignancy—the more malignant the tumor, the less typical the cytoplasmic structure.

My own examination with the electron microscope has demonstrated that, in fact, the cytoplasmic structures of differentiation are optimally differentiated in the epidermis at the stage of hyperplasia, and that in the transition from hyperplasia to carcinoma these structures diminish and in truly developed carcinoma are completely absent in the most dedifferentiated part of the tumor (*8*). It should be emphasized that from this viewpoint the criterion for cytoplasmic differentiation is the occurrence of tonofibrils, whereas intercellular cytoplasmic bridges without tonofibrils are not necessarily a product of differentiation—as demonstrated in figures 14 to 16.

Adolph, Baker, and Leiby (*3*) could find no tonofibrils in their electron microscopic study of normal human skin, and they assume, therefore, that tonofibrils are artefacts. They found only intercellular bridges. I do not think that it is justified, in view of technically rather poor photomicrographs, to deny facts which have been definitely established by ordinary light microscopy, as is the case with tonofibrils. To give definite proof, it should be mentioned here that it is easy to demonstrate the presence of tonofibrils in the unfixed human epidermis by means of phase-contrast microscopy [von Albertini (*4*)].

Gessler *et al.* (*5*), in figure 8 of their second publication, have shown a squamous-cell epithelioma with very pronounced fibril systems. We have made similar findings in the so-called senile carcinoma of the skin [von Albertini (*6, 7*)] which, however, has been shown to have a low degree of malignancy. The findings of Gessler *et al.* are not contradictory to ours.

Summary

Electron microscopic histologic and cytologic studies of the development of hyperplasia and carcinoma in the epidermis of Swiss and albino mice, following painting with methylcholanthrene, confirmed the author's earlier opinion (*8*) based on phase microscopy that the development of a malignancy involved both qualitative and quantitative disorganization of the cytoplasm and subsequent pronounced loss of differentiation, with

diminished cytoplasm. The electron microscope demonstrated the gradual parallelism between loss of cytoplasmic structure and degree of malignancy. Structurally, the nuclei do not seem to take part in the disorganization but rather show increase in vital substances. The last stage of carcinogenesis resulted in the formation of a highly anaplastic dedifferentiated spindle-cell carcinoma. Tonofibrils were not found in the dedifferentiated cells of the malignant carcinoma.

The electron microscope is a very important method in the evolution of our knowledge of the histopathology of tumors.

Grateful acknowledgment is made to Dr. E. V. Cowdry, of Washington University, for his hospitality during my stay as visiting professor in his department.

I am equally indebted to Dr. R. W. G. Wyckoff, of the Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, for extending his hospitality to me in the form of prolonged permission to use his electron microscope. His coworkers, Messrs. Mosley and Noda, have given me much help and assistance. I am grateful to Dr. H. B. Andervont, of the National Cancer Institute, for the supply of his experimental animals and to Dr. H. L. Stewart, of the same institution, for his hospitality.

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PLATE 147

FIGURE 1.—Normal epidermis of mouse skin. Ordinary light miscroscopy. Hematoxylin and eosin. × 240

FIGURE 2.—Hyperplastic epidermis with well-developed tonofibrils and distinct intercellular bridges. In some places these bridges are crossed by tonofibrils and, therefore, enlarged (so-called desmosomes). Mouse #805. Electron microscopy. Osmium fixation. × 7,400 (approx.)

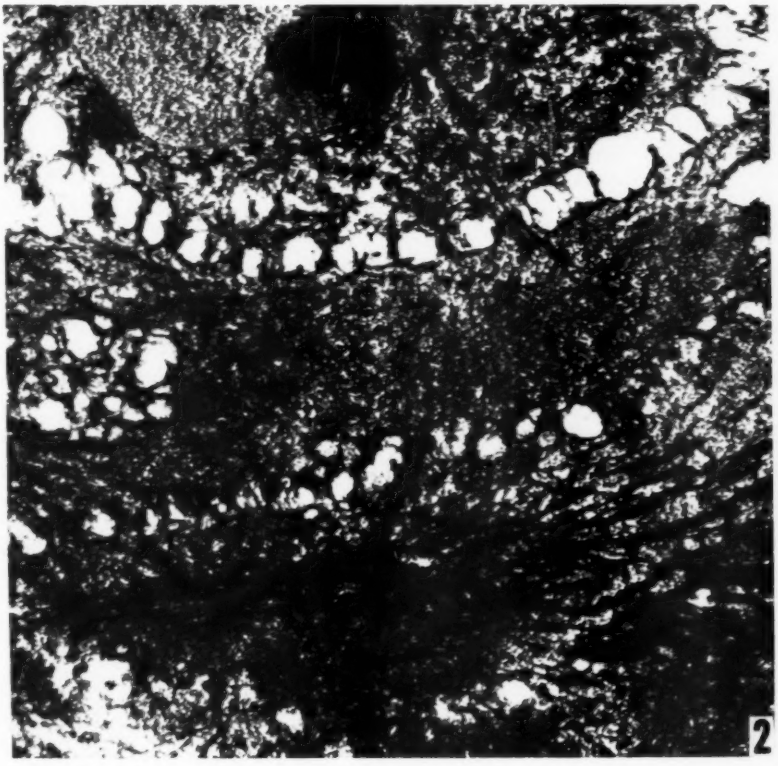

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PLATE 148

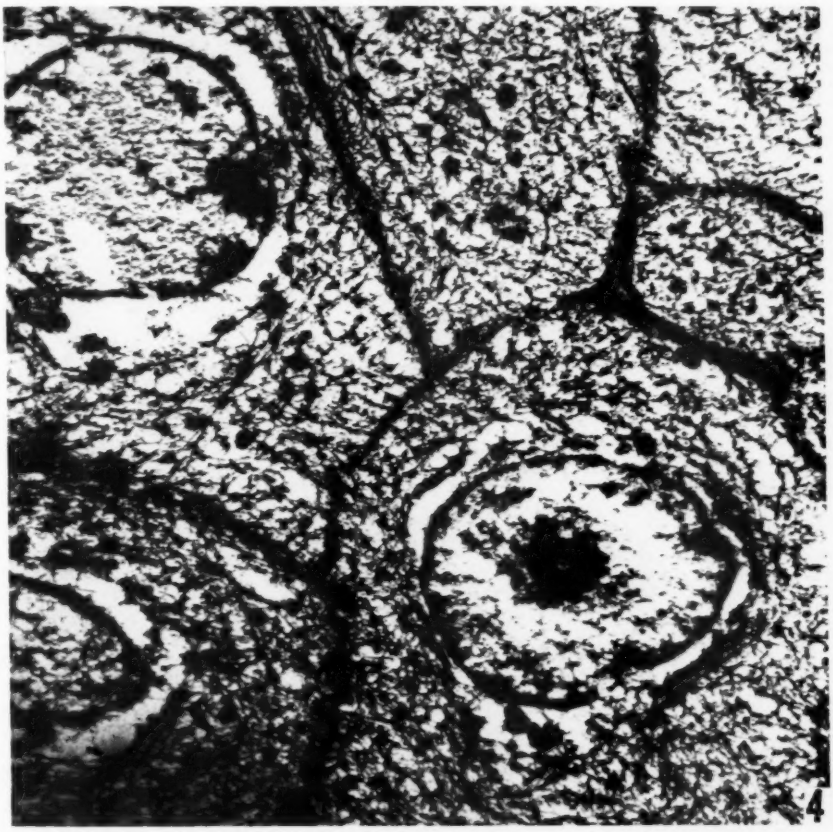
FIGURE 3.—Hyperplastic epidermis. Layers of the epidermis with beautiful tonofibril systems. Intercellular bridges are replaced by desmosomes. Mouse #805. Electron microscopy. Osmium fixation. × 6,400 (approx.)

3

PLATE 149

FIGURE 4.—Hyperplastic epidermis, from the upper epidermal layers. Distinct cellular borders, formed by a close approximation of tonofibrils and interposition of desmosomes. Mouse #408. Electron microscopy. Osmium fixation. × 9,000 (approx.)

FIGURE 5.—Hyperplastic epidermis with transition into carcinoma. Pronounced broadening and polymorphism ("restlessness") in the stratum germinativum. Disorganization of nucleo-cytoplasmic relationship. Mouse #408. Light microscopy. Bouin fixation. × 360



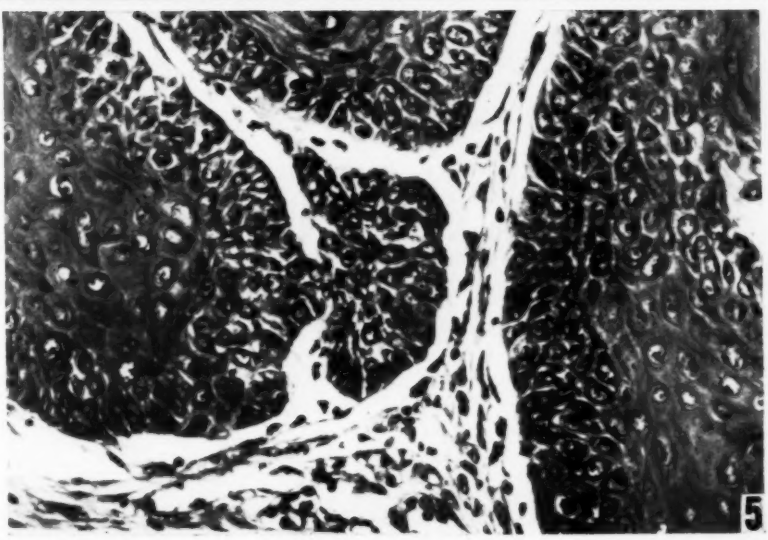


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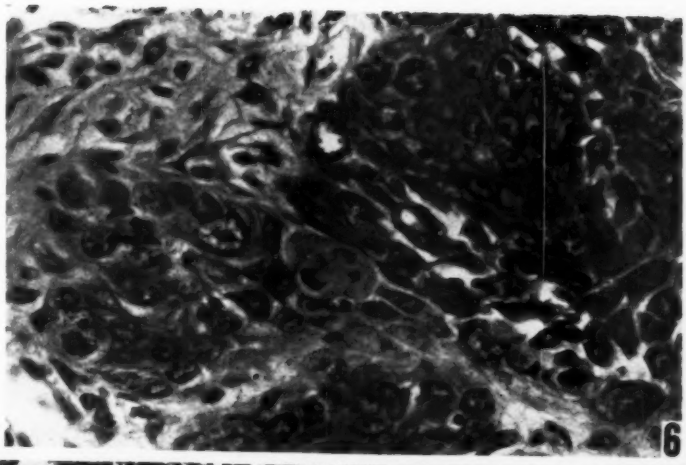
PLATE 150

FIGURE 6.—Segregation and disorganization of the cell layers in the stratum germinativum. Spindle cells with sparse cytoplasm appear. Atypical mitoses. This mouse was painted 15 times with 0.6 percent methylcholanthrene solution. Light microscopy. Bouin fixation. × 480

FIGURE 7.—Pronounced broadening of the intercellular spaces, with reduction of cytoplasm and of its structures. Primitive protoplasm, and intercellular bridges; rare tonofibrils. Mouse #408. Electron microscopy. Osmium fixation. × 7,000 (approx.)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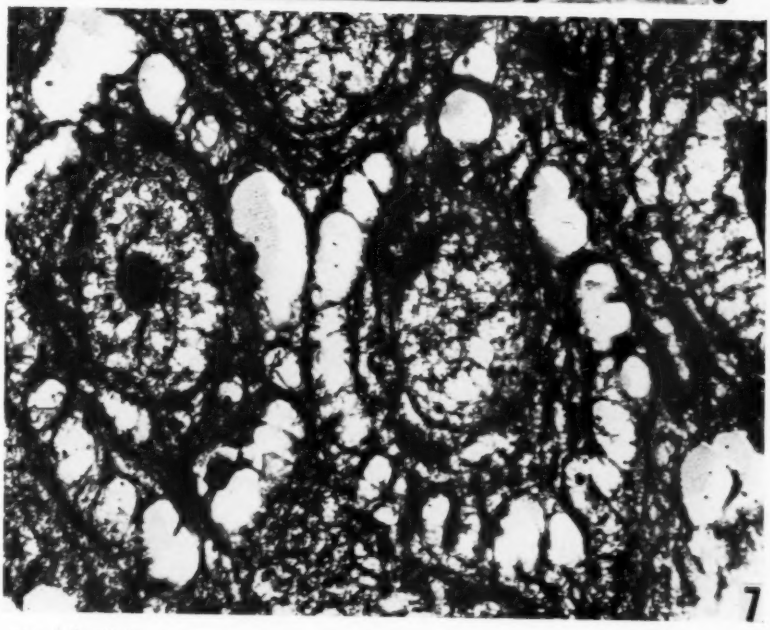
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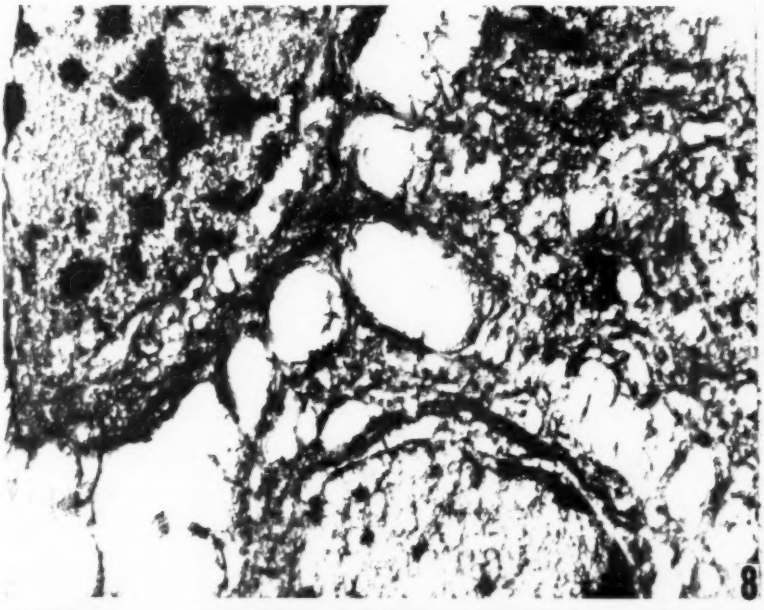
PLATE 151

FIGURE 8.—Same findings as in figure 7, but even more atypical and more pronounced. Mouse #408. Electron microscopy. Osmium fixation. × 8,500 (approx.)

FIGURE 9.—Almost complete loss of cytoplasm. Only a few protoplasmic bridges remaining, which extend from nucleus to nucleus. Mouse #408. Electron microscopy. × 9,000 (approx.)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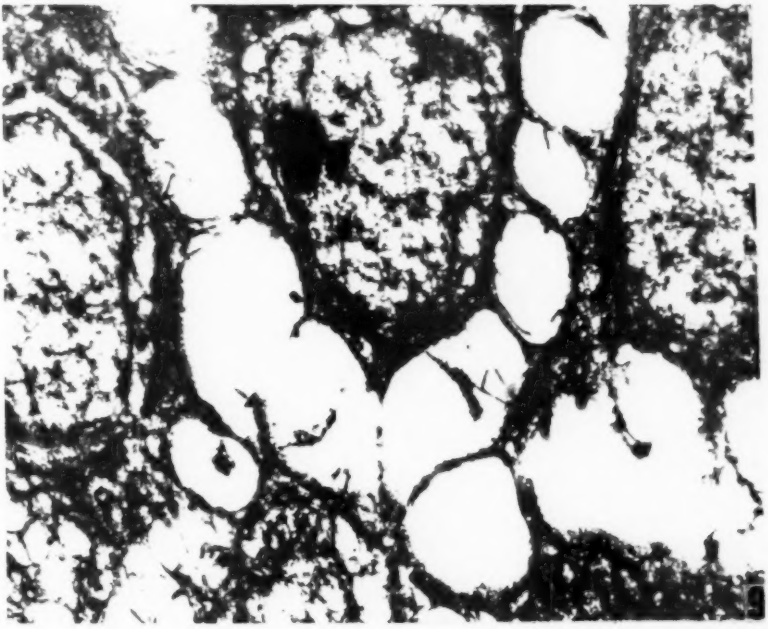
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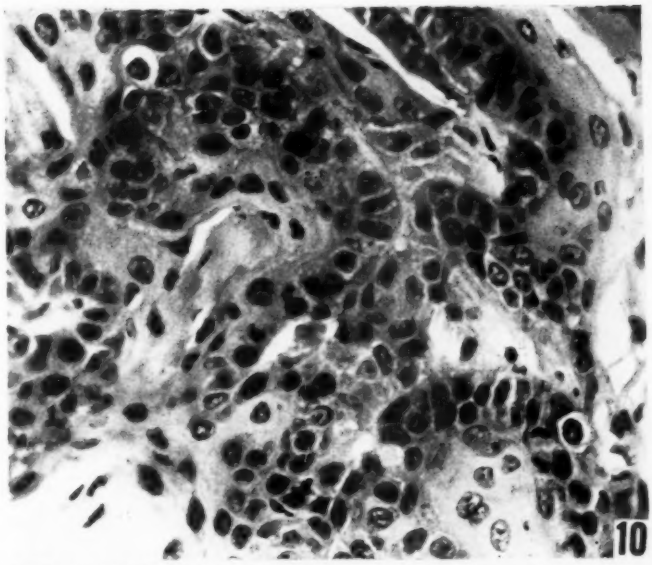
PLATE 152

FIGURE 10.—Slightly dedifferentiated transplantable squamous-cell carcinoma. Mouse of strain C57L. Light microscopy. Bouin fixation. × 360 (approx.)

FIGURE 11.—Transplantable squamous-cell carcinoma. Intercellular bridges are present, but the tonofibrils are indistinct. Electron microscopy. Osmium fixation. × 8,200 (approx.)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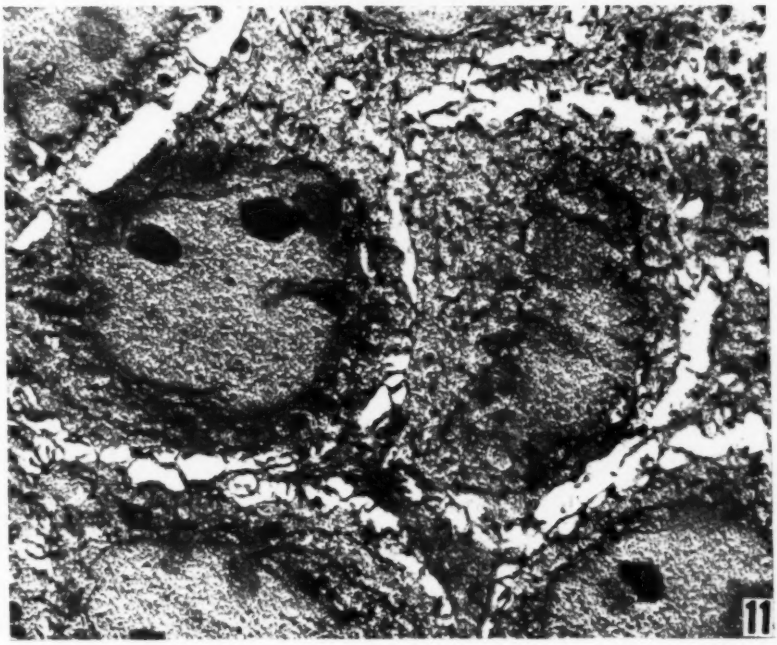
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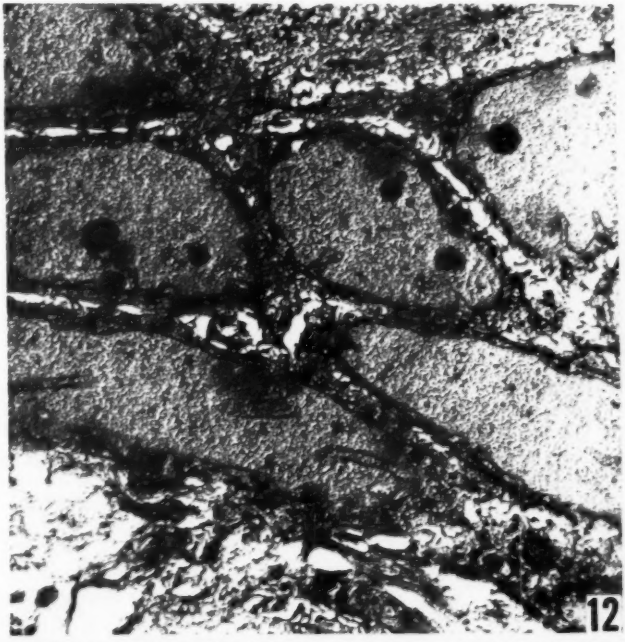
PLATE 153

FIGURE 12.—Same specimen as figures 10 and 11. Example of the tight packing of nuclei and pronounced reduction of cytoplasm. Electron microscopy. Osmium fixation. × 7,000 (approx.)

FIGURE 13.—Highly dedifferentiated (metastasizing) squamous-cell carcinoma with spindle cells and small islands of epidermal dedifferentiation. Mouse #626. Light microscopy. Bouin fixation. × 360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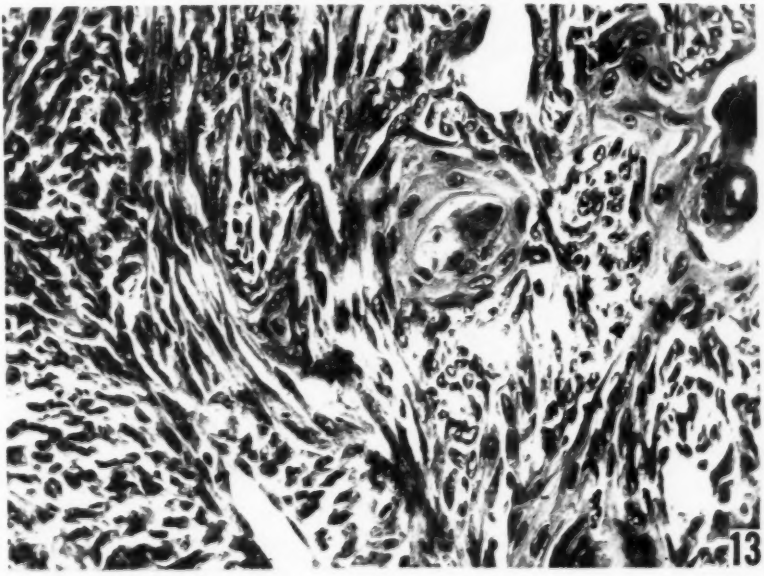
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PLATE 154

FIGURE 14.—Anaplastic cells, with complete lack of cytoplasmic structure. The nuclei, however, appear well preserved. Mouse #626. Electron microscopy. Osmium fixation. × 9,000

FIGURE 15.—Same as figure 14. Osmium fixation. × 15,000

FIGURE 16.—Same as figure 14. Osmium fixation. × 9,000

14

15

16

Tumors of the Urinary Bladder, Gall Bladder, and Liver in Dogs Fed *o*-Aminoazotoluene or *p*-Dimethylaminoazobenzene[1,2]

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This paper deals with the production of tumors in the urinary bladder and in the gall bladder and liver of dogs by feeding either *o*-aminoazotoluene or *p*-dimethylaminoazobenzene. These substances are known to produce tumors in rats and mice, but a carcinogenic action in dogs has, to our knowledge, not been previously reported.

Materials and Methods

The animals used were young adult dogs of both sexes, some Irish terriers and some mongrels. Their diet was a commercial dry chow in pellet form, occasionally supplemented by cod-liver oil. The chemicals were administered either in dry form or in corn-oil solution, in gelatin capsules. Table 1 outlines the several experimental situations used. Tissues taken at autopsy were fixed in 10 percent formalin, and in some instances in Bouin's or Zenker's fluids; paraffin sections were stained with hematoxylin and eosin.

TABLE 1.—*Treatment of dogs*

Agent used	Dose, (*mg./kg./day*)	Number of dogs	Number with tumors	Remarks
o-Aminoazotoluene...	20	5	0	All died within 8 weeks, principally from liver damage.
"	5	5	4	The one dog without tumor was fed for 4 months, the others for 30–62 months.
p-Dimethylaminoazobenzene.	20	10	2	The 8 dogs without tumor died within 16 months.
"	5	9	0	Six of the 9 dogs were fed for 63 months.

[1] Received for publication November 13, 1952.
[2] We wish to thank the Laboratory of Pathology of the National Cancer Institute for preparation of the photographs.

Results

Table 2 gives the salient facts concerning the tumors found in six dogs, four of which had received *o*-aminoazotoluene and two *p*-dimethylaminoazobenzene. Although the number of animals involved is too small to be statistically significant, it may be seen from the table that *a*) liver and gall-bladder tumors occurred only with *o*-aminoazotoluene; *b*) these and urinary-bladder tumors did not occur together; *c*) more portal cirrhosis was present without liver tumor formation than with such tumor; *d*) there was no sex difference in tumor incidence. However, one possible sex difference is that the tumors of the urinary bladder were larger in the two males than in the two females.

Tumors of the Urinary Bladder

Gross description will be limited to a few sentences in addition to the brief notes in table 2, since a gross or low-power microscopic photograph of each urinary bladder (figs. 1–4) is also presented. All tumors were soft in consistency, and were whitish in color in the two dogs fed *o*-aminoazotoluene and pinkish in the two others. In only one bladder was the tumor markedly invasive; close inspection of the lower center of figure 4 will reveal invasion to the serosa, which was noticeable at autopsy. This tumor also invaded the superior portion of the prostate, and the periurethral lymphatics to below the prostate. In the remaining 3 bladders invasiveness was perhaps debatable at very low magnification (figs. 2 and 3), but it was definite although slight at higher magnification. Microscopically, all tumor masses sectioned were, with one exception, epithelial. The exception was the largest nodule shown in figure 4; all but the surface of this particular nodule had a sarcomatous appearance, and its classification, as between carcinoma with spindle-shaped cells and actual sarcoma, was held to be an open question by several consultants. The remaining, epithelial, tumors retained more or less closely the appearance of normal urinary tract epithelium. A field from the center of the papilloma in figure 3 is a representative average and is shown in figure 5. There was, in places, a slight degree of epidermoid change. Mitotic figures were present in moderate number in all of the tumors.

Tumors of the Liver and Gall Bladder

Two dogs, neither having urinary-bladder involvement, had tumors involving both the liver and the gall bladder (table 2). The gall bladder and the adjacent liver and main bile ducts were definitely the chief focus of tumor proliferation in both instances, even though in one dog there was much additional tumor. Whether the tumors arose primarily in the liver, the large bile ducts, or the gall bladder, or whether they arose independently in more than one of these locations, was difficult to judge both on gross examination and microscopically, as may be seen to some extent in the low-power photomicrograph (fig. 6). Microscopically, the tumor in this animal was a relatively undifferentiated carcinoma, com-

TABLE 2.—*Data on dogs having tumors. The first four animals received o-aminoazotoluene, 5 mg./kg./day, and the last two received p-dimethylaminoazobenzene, 20 mg./kg./day, orally.*

Dog No.	Sex	Age (*months*)	Months on experiment	Tumors in urinary bladder	Hepatic cirrhosis	Tumors in liver	Tumors in gall bladder	Histologic classification of tumors
V-108...	M	50	30	About 12 papillary masses, up to 1 cm. diameter.	Marked....	.....	.....	Carcinoma with local extension.
20-84*...	F	62	40	.....	Slight.....	7 × 4 × 2½ cm., plus about 50 scattered nodules.	Continuous with that in liver.	Adenocarcinoma.
32-103...	F	56	43	Several flat mucosal elevations, up to 12 × 8 × 4 mm.	Marked....	.....	.....	Early carcinoma.
16-54....	M	87	62	.....	Slight.....	3-cm. rounded mass plus smaller irregular foci, near gall bladder.	Small papillae and flat nodules.	Adenocarcinoma in gall bladder; cholangioma and hepatoma in liver.
29-98....	M	57	†48	Filled with papillary tumor.	Slight.....	.....	.....	Papilloma with beginning invasion.
35-123...	F	44	38	2 papillomas; largest 12 × 8 × 6 mm.	Moderate...	.....	.....	Papilloma with beginning invasion.

*This dog also had extensive peritoneal carcinomatosis.
†No chemical administered for last 32 months; only dog so treated.

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posed of irregular masses of rather small cells with moderately hyperchromatic and usually oval nuclei, and little cytoplasm (fig. 8). Glandular formations of biliary type were frequent in the gall-bladder tumor, less definite and less frequent in the liver, and poorly formed in the peritoneal implants. Mitotic figures were numerous. In the dog with the smaller amount of tumor, the main and the smaller liver tumor masses were adjacent to the gall bladder (fig. 7), and small flat nodules and papillary masses were present in the neck of the gall bladder and in the cystic duct. A section of the tumor in the neck of the gall bladder showed definite adenocarcinoma; that in the liver was partly hepatoma (fig. 9) and partly cholangioma (fig. 10). Mitotic figures were moderate in number.

Discussion

The literature on tumors of the liver, gall bladder, and urinary bladder in the dog, either spontaneous or induced by various agents, will not be reviewed here because of its extent. The same reason applies even more forcefully to the extensive literature on the production of tumors by the two dyes used in these experiments in other animal species. The spontaneous dog tumors are discussed by Mulligan (*1*), and both the spontaneous and induced by Willis (*2*). A very recent paper in this journal (*3*) concerns tumors of the liver and urinary bladder produced in dogs by the feeding of 2-acetylaminofluorene.

As control material, several hundred of our control and experimental dogs fed other substances have shown no tumors of the liver, gall bladder, or urinary tract. Not all of these dogs had been observed for as long a time as those reported here, but many of them had. The tumors that were seen included one each of adenoma of the thyroid gland, papilloma of the ovary, argentaffinoma of the small intestine, fibropapilloma of the jaw, and carcinoma of the breast. All of these tumors were 2 cm. or less in diameter.

The two dyes that produced the dog tumors reported here were not the items of primary interest in the study of which this report forms a part. They were included as "positive controls" among 40 other dogs fed a total of 9 different aromatic amines (studied because they were possible food contaminants) and food dyes, whose chronic toxicity was being investigated. Among the 40 other dogs, fed for periods of 33 to 74 months, no tumors whatever were found.

Summary

Of 5 dogs fed 5 mg./kg./day of *o*-aminoazotoluene, one died without tumor after 4 months; the others were fed for 30 to 62 months, and all 4 of these had tumors. Two had tumors in the urinary bladder and two had tumors in the liver and gall bladder. At a dosage level of 20 mg./kg./day of the same compound, all of 5 dogs died within 8 weeks, principally from liver damage, without tumor.

Of 10 dogs fed 20 mg./kg./day of *p*-dimethylaminoazobenzene, 8 died within 16 months, without tumor. The remaining 2 showed bladder

tumors after 38 and 48 months, respectively. At a dosage of 5 mg./kg./day of the same compound, none of 6 dogs fed for 63 months, and none of 3 dying earlier, had tumors.

Considering together all animals with tumor, 4 had tumors of the urinary bladder which were epithelial, and varied from borderline to moderate in histologic malignancy. Extension beyond the bladder was present in one instance. Two others had tumors of the liver and gall bladder, showing several types of histologically malignant epithelial tumor. Peritoneal carcinomatosis was present in one instance. What the exact site of origin of the liver and gall-bladder tumors was, or whether they arose in more than one site, is uncertain.

To our knowledge, production of tumors in dogs with these two agents has not been previously reported.

References

(*1*) MULLIGAN, R. M.: Neoplasms of the Dog. Baltimore, The Williams & Wilkins Co., 1949. Pp. 4, 5, 111, 113.

(*2*) WILLIS, R. A.: Pathology of Tumours. London, Butterworth & Co., 1948. Pp. 94, 95, 433, 439, 470.

(*3*) MORRIS, H. P., and EYESTONE, W. H.: Tumors of the liver and urinary bladder of the dog after ingestion of 2-acetylaminofluorene. J. Nat. Cancer Inst. 13: 1139–1165, 1953.

PLATE 155

FIGURE 1.—Photograph, actual size, of the extensive papillary tumor in the opened urinary bladder of Dog 29-98. The prostate gland is at the right.

FIGURE 2.—Section through the largest of the several mucosal elevations in the urinary bladder of Dog 32-103. Beginning invasion of the lamina propria may be seen at X, and is evident elsewhere with higher magnification. × 8

FIGURE 3.—Section through the largest of the two papillary tumor masses in the urinary bladder of Dog 35-123. The ragged edge of the tumor at the base of the stalk is shown. Examination of the mucosa, especially in the lower half of the section, will show several small foci of epithelial proliferation. × 7

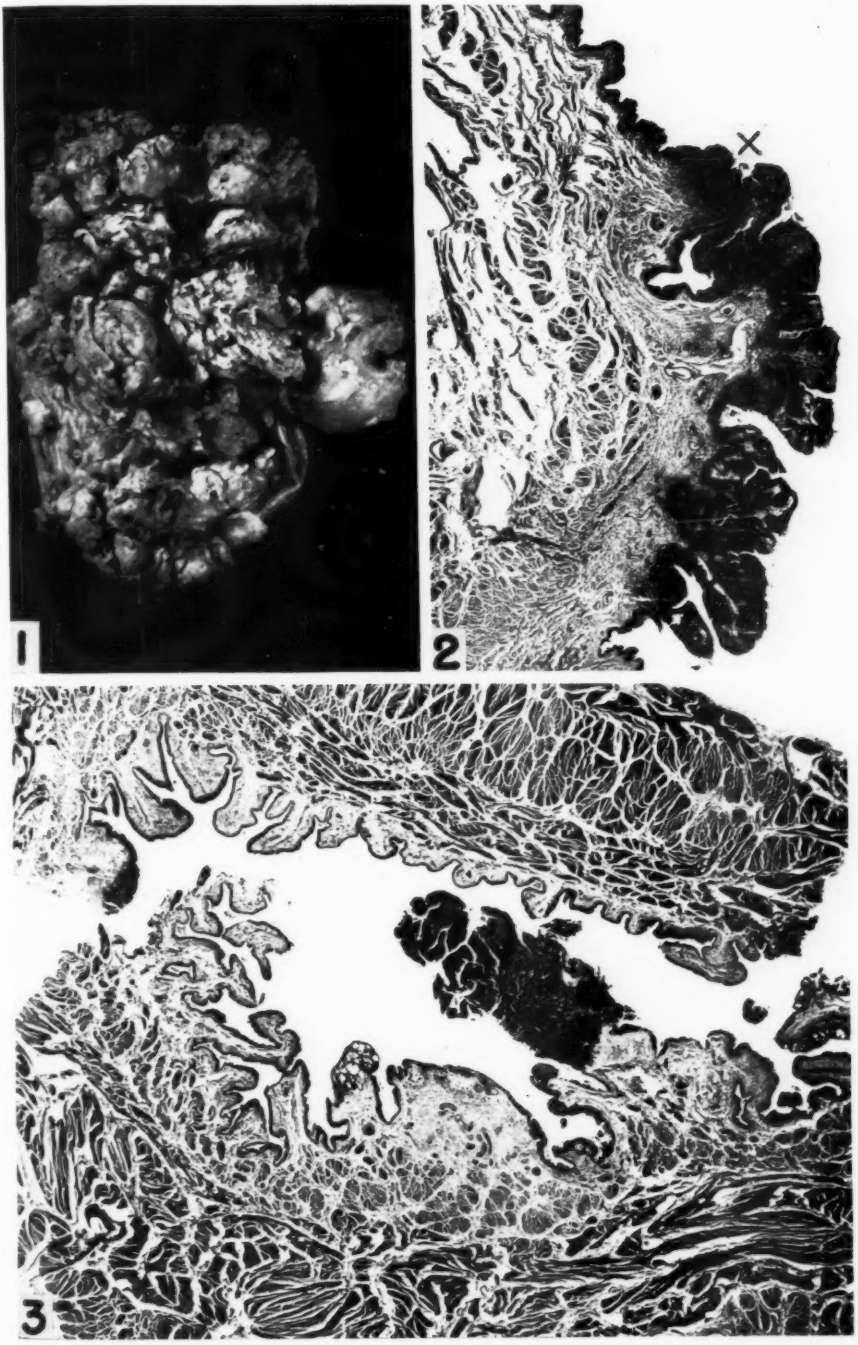

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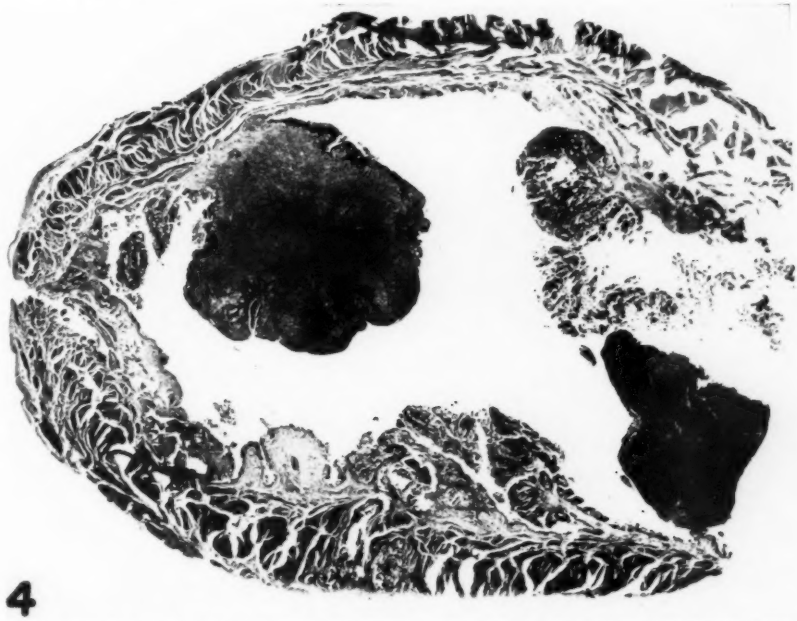
PLATE 156

FIGURE 4.—Cross section of the greater portion of the urinary bladder of Dog V-108, showing several of the numerous tumor masses. Inspection of the lower central portion of the photomicrograph will reveal tumor penetrating the bladder wall to the serosa. × 4

FIGURE 5.—Section from the central portion of the papillary tumor shown in figure 3. Note the relatively close resemblance to the normal transitional epithelium of the urinary tract, and the sparse stroma. × 380



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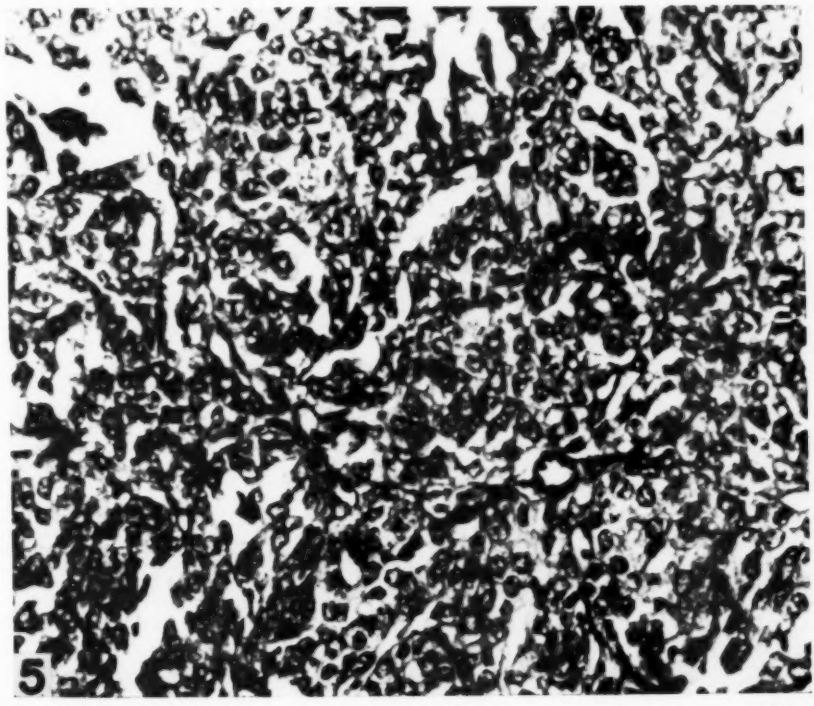

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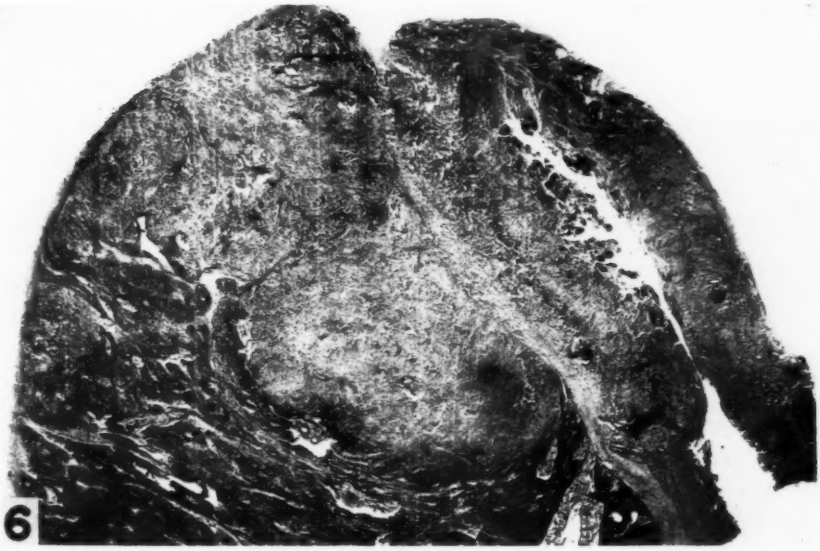
PLATE 157

FIGURE 6.—Low-power photomicrograph of tumor in the fundus of the gall bladder and in the adjacent liver of Dog 20-84. The narrow pale stripe running from the notch in the top center of the photo to the lower right is the fibrous tissue of the gall-bladder wall and liver capsule. × 7

FIGURE 7.—Low-power photomicrograph of a portion of tumor in the liver of Dog 16-54. In addition to the encapsulated, lobulated, and slightly cystic tumor mass in the right half of the photo, small tumor foci (somewhat larger ones were also present) may be seen near the gall bladder, and the mucosa of the gall bladder is thickened by epithelial proliferation. × 4

FIGURE 8.—Tumor from mass in liver shown in figure 6. Relatively undifferentiated epithelial tumor, with partial glandular formation seen at right. × 380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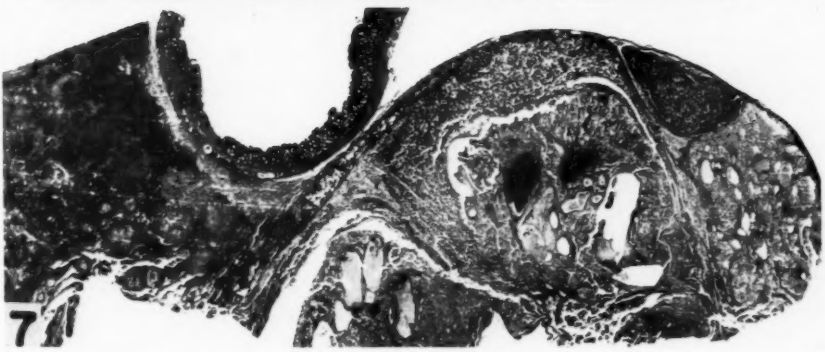
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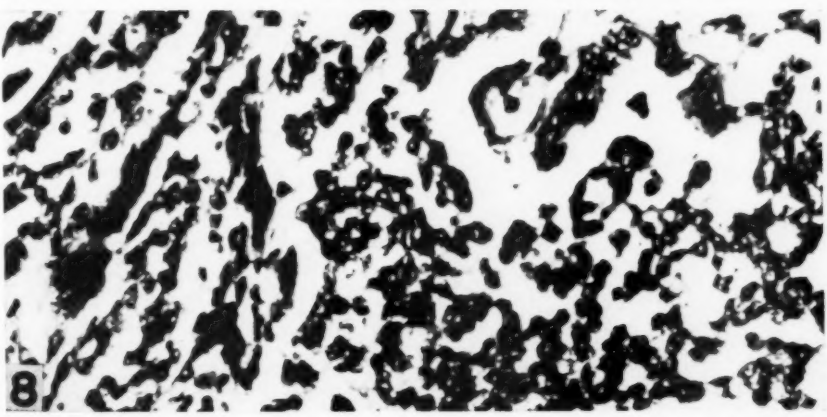
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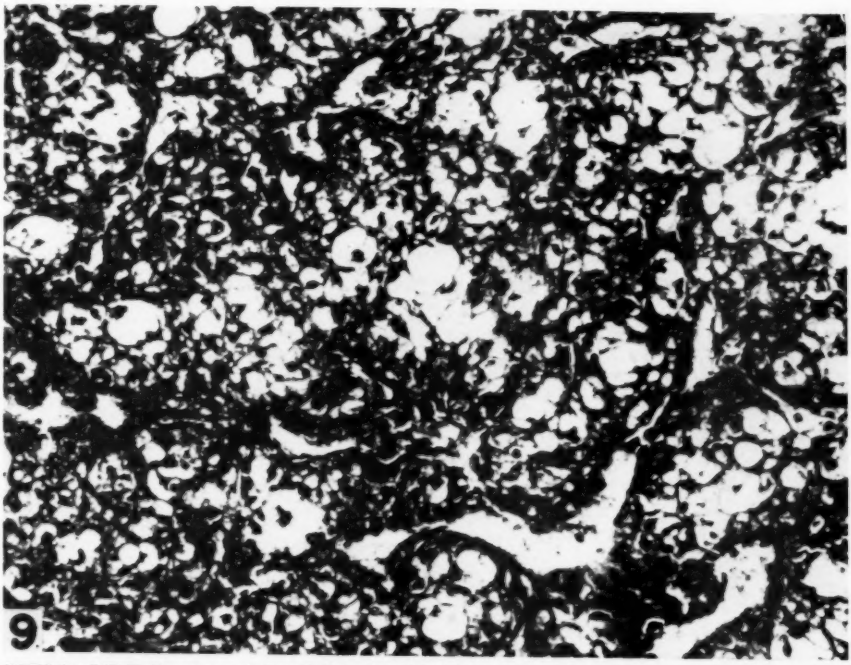
PLATE 158

FIGURE 9.—Hepatomatous type of tumor from the larger mass shown in figure 7, from Dog 16-54. × 200

FIGURE 10.—Cholangiomatous type of tumor, also from the liver of Dog 16-54. × 200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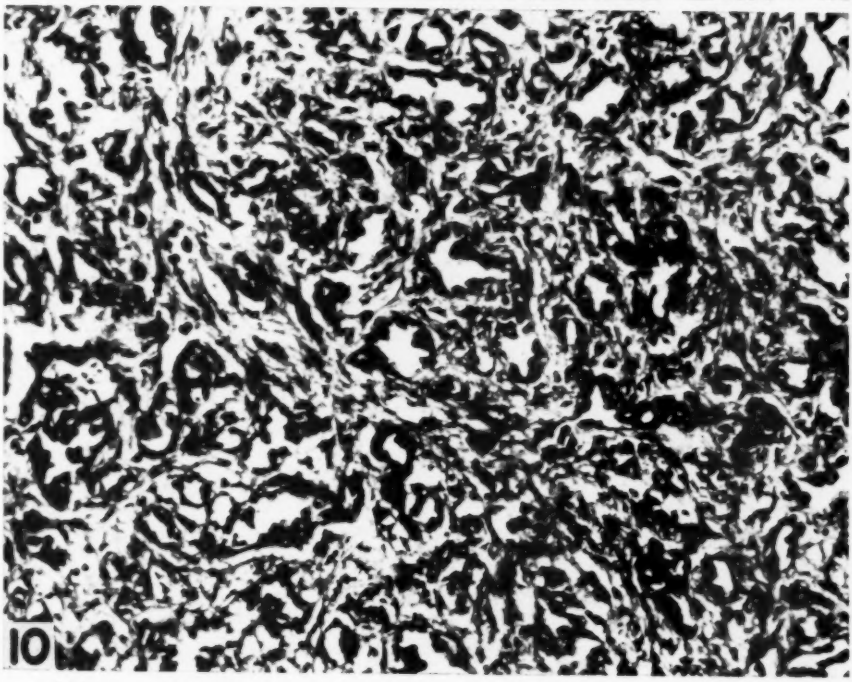

10

A Cytosieve Permitting Sterile Preparation of Suspensions of Tumor Cells for Transplantation[1, 2]

GEORGE D. SNELL,[3] *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*

One of the common methods of transplanting tumors is by subcutaneous injection of tumor cell suspension. Cell suspensions for this purpose have been prepared in a variety of ways. For example, Craigie (*1*) has described a press for "rapid reduction of tumor tissue to a suspension of fine fragments and separated cells."

The "cytosieve" described herein has now been used in this laboratory for the preparation of tumor cell suspensions for transplantation for more than a year, and has given extremely satisfactory results. It can be constructed at moderate initial cost, is easy to operate, gives a suspension predominantly of single cells and entirely free from clumps capable of clogging a needle, and greatly reduces the chances of bacterial contamination during the various steps involved in transplantation.

The parts of the cytosieve are illustrated in text-figure 1. The essential element is an acid-resistant, stainless steel cylinder with a fine wire screen inserted in one end. The screen rests on a ledge cut in one end of the cylinder and is held permanently in place by a tightly fitting ring of stainless steel. The screen used has the following manufacturer's specifications: material 18/8 stainless, mesh 100 × 100, wire size .0045.

The only other part requiring special manufacture is a Pyrex glass tube, 22 mm. outside diameter, with one end formed to receive a serum bottle stopper.[4]

The elements of the cytosieve are assembled in the order: serum bottle stopper, glass tube, heavy rubber tube, sieve (with sieve end away from the glass), thin-walled rubber tube, clamp, and cotton stopper. The rubber stopper is covered with a piece of aluminum foil, and the whole unit autoclaved.

For use, the cotton stopper is removed and a piece of tumor tissue inserted in the thin-walled tube. The size of the tissue may vary from

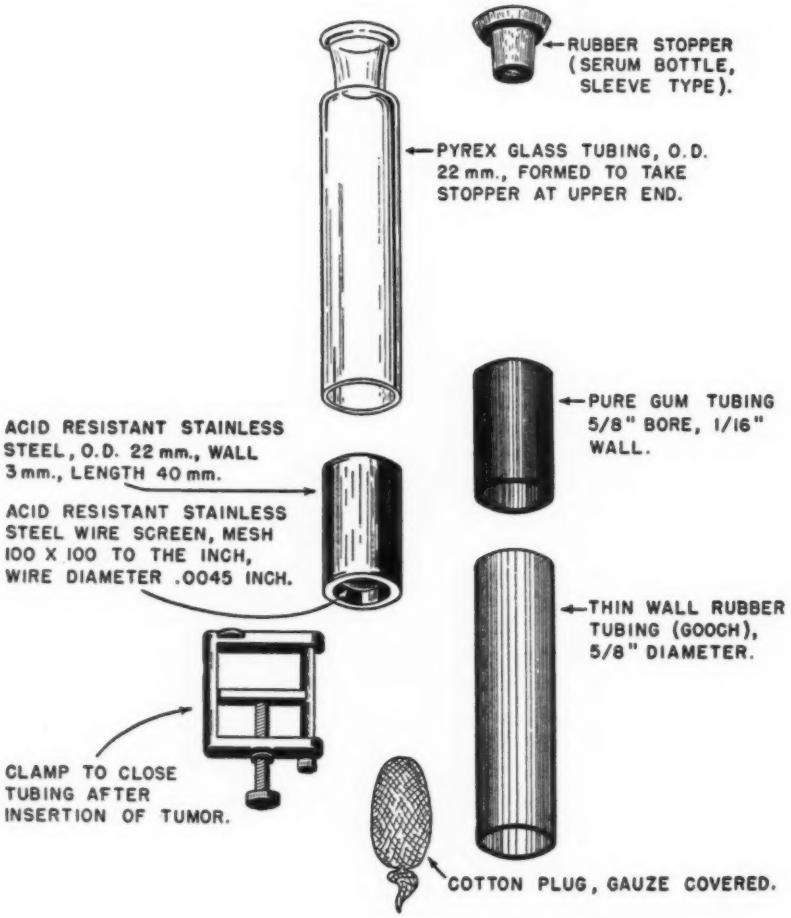
[1] Received for publication March 6, 1953.

[2] This investigation was supported by research grant C-1329 (C2) from the Nationa Cancer Institute, National Insitutes of Health, U. S. Public Health Service, and by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[3] The author gratefully acknowledges the technical assistance of Miss Priscilla Smith.

[4] Glass tube and stainless steel sieve were prepared for us by Macalaster Bicknell Co., 243 Broadway, Cambridge, Mass.

the size of a small pea to 1 cubic centimeter. The end of the tube is immediately closed with the clamp and 2 to 10 ml. of medium inserted with a hypodermic syringe through the serum bottle stopper. If more than 5 ml. of medium is added it is usually advisable to withdraw some air before the syringe is removed. With the apparatus held glass end up, the thin-walled tube is kneaded and the tissue and medium worked back and forth between the fingers. With most tumors only a brief kneading is necessary. The tube is then inverted and the cell suspension forced back through the sieve with gentle pressure. If a tumor contains much connective tissue, or if several lots of suspension are to be prepared from one loading of tumor, it is advisable to let the suspension settle for a minute, and then to pinch off the lower end of the tube with thumb and forefinger before inverting.


RUBBER STOPPER (SERUM BOTTLE, SLEEVE TYPE).
PYREX GLASS TUBING, O.D. 22 mm., FORMED TO TAKE STOPPER AT UPPER END.
PURE GUM TUBING 5/8" BORE, 1/16" WALL.
ACID RESISTANT STAINLESS STEEL, O.D. 22 mm., WALL 3 mm., LENGTH 40 mm.
ACID RESISTANT STAINLESS STEEL WIRE SCREEN, MESH 100 X 100 TO THE INCH, WIRE DIAMETER .0045 INCH.
THIN WALL RUBBER TUBING (GOOCH), 5/8" DIAMETER.
CLAMP TO CLOSE TUBING AFTER INSERTION OF TUMOR.
COTTON PLUG, GAUZE COVERED.

TEXT-FIGURE 1.—The cytosieve: parts and specifications.

This keeps connective tissue and any lumps of tumor from reaching and clogging the screen. The suspension is removed through the stopper with a hypodermic syringe. As the rubber tubing gets old it may cease to fit tightly, with resulting leakage. This is usually a problem only when several lots of cell suspension are to be taken from one loading of tumor. We keep several clarinet mouthpiece clamps for use on such occasions. These are slightly too loose as purchased; placing solder on the inside reduces them to the correct size. The taper of this type of clamp just accommodates the unequal circumferences produced by the two thicknesses of rubber tubing. For routine transplants where 4 or 5 ml. of cell suspension is adequate, the clamps are not necessary.

After experimenting with several types of medium, we have settled on a buffered glucose-Ringer's solution containing 1 ml. of McIlvaine's buffer (*p*H 7.2) and 1 ml. of stock glucose solution (600 mg./ml.) for every 100 ml. of medium. This was adopted, with slight modification, from Kalfayan and Kidd, and Toolan (*2, 3*).

Cell suspensions prepared with the cytosieve consist predominantly of single cells and lend themselves readily to counting in a hemocytometer. The number of obviously damaged cells varies with the tumor. Most cells from sarcomas and leukemias, as observed in the counting chamber, appear normal; damage is perhaps more common with mammary carcinomas. Hewitt (*4*) has reported that tumor cell suspensions prepared by mincing and sedimenting contain 80 to 90 percent nonviable cells as judged by trypan-blue staining and study under the phase-contrast microscope. We have not attempted to examine our preparations by these methods.

Suspensions prepared with the cytosieve usually contain between 2,000,000 and 10,000,000 cells per ml. Counts up to 25,000,000 cells per ml., or more, are obtainable with leukemias. One large loading of tumor and 5 or 6 successive loadings of medium can be made to yield 25 ml. of suspension with a count of 4,000,000 cells per ml.

Several tests have been run to determine the behavior of tumors transplanted by this method.

In one experiment the relation of dosage to number of mice growing the tumor was tested. BALB/cSn mice were inoculated subcutaneously with BALB/c fibrosarcoma S621/Sn-S [5] in doses ranging from 100 to 102,400 cells per mouse. The results are given in table 1. It will be seen that 6,400 cells per mouse gave 90 percent, and 25,600 cells per mouse gave 100 percent positive growths. With several other sarcomas the minimum dose giving 100 percent mortality is in the neighborhood of 10,000 cells. Leukemias seem to require a lower dosage level, and some new tumors and perhaps some mammary carcinomas a higher one.

[5] We are using for our transplantable tumors a system of nomenclature borrowed from the nomenclature system developed for inbred strains of mice (*5*). "S621" is the basic designation of the tumor, the "Sn" refers to a line carried by Snell, the "S" indicates a line carried by cell suspension.

TABLE 1.—*Fraction of BALB/cSn mice growing tumor S621/Sn-S following inoculation at varying dosage levels*

Number of cells per mouse....	100	400	1, 600	6, 400	25, 600	102, 400
Fraction of mice dying.....	0/20	2/18	2/19	18/20	20/20	16/16

Another test involved the effect of standing on the viability of the tumor cell suspension. It will be seen from the results in table 2 that suspensions of Sarcoma I tested in strain A hybrids showed only a very small drop in fraction of positive growths after standing for 5 hours at room temperature. Results were essentially similar with suspensions that had stood in the refrigerator for 5 hours, or in medium with added oxygen (data not given).

TABLE 2.—*Effect of standing 5 hours at room temperature on fraction of positive growths obtained with Sarcoma I in strain A hybrids. Combined results of three experiments.*

When injected	Cells per mouse	Fraction dying
Fresh	5, 000	28/30
Same	1, 000	23/29
After 5 hrs. standing at concentration of 50,000 cells per ml....	5, 000	26/29
Same	1, 000	19/30
After 5 hrs. standing at concentration of 4,000,000–9,000,000 cells per ml	5, 000	19/20
Same	1, 000	16/19

The transplant interval for tumors transplanted by cell suspension is usually slightly longer than for tumors transplanted by trocar. However, we have noted with some tumors a tendency for the interval to shorten during the course of the first few transplants. This is of course expected with new tumors, but it seems to occur also, though to a lesser degree, with some tumors that have passed through many generations of trocar transplantation. Possibly this means that a selection of cell strains occurs whereby there is an adaptation to the new element in the tumor's environment.

One of the principal objectives sought in developing the cytosieve was a reduction in the possibility of bacterial contamination during transplantation. That this was acheived is indicated by the fact that we have never had a pus-forming infection in a tumor transplanted by this method, whereas such infections do sometimes occur in tumors carried by trocar. Of course the possibility of picking up infections from the blood stream of the host cannot be ruled out.

Summary

A "cytosieve" for preparing suspensions of tumor cells for transplantation is described. The suspensions consist predominantly of single cells. Sterile conditions are easily maintained.

The relation of number of cells inoculated to number of positive growths was investigated, using fibrosarcoma S621 and BALB/c hosts. A Sarcoma

I cell suspension lost only a small part of its activity after standing 5 hours.

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